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EFFECT OF PREVIOUS COLD STORAGE ON THE RESPIRATION OF VEGETABLES AT HIGHER TEMPERATURES[1]

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INTRODUCTION

It is common practice to hold fresh vegetables at low temperatures in order to retard the processes responsible for the deterioration and spoilage of the product. Much experimental work has been done to determine the storage conditions most suitable for different vegetables, but less attention has been given to the effect of the previous storage temperature on the physiology and keeping qualities of particular vegetables at market temperatures.

The high initial respiration rate previously found in potatoes (*9*)[2] following transfer from cold storage to higher temperatures is no doubt an important factor in the keeping qualities of such potatoes at market temperature, unless adequate ventilation is provided during this critical period. The effect of this respiratory response after a period of low-temperature storage would also apply to potatoes at the higher temperatures of late common storage if the temperature during the winter months had fallen below 5° C. for a period of 3 weeks or more. It is of both practical and scientific importance to know whether a high respiration rate for a time after cold storage is a common response of fleshy vegetables. This paper reports the results of experiments made to determine the respiratory responses at 22° of the following vegetables after various periods in storage at 2.5°: Potatoes (*Solanum tuberosum* L.), sweetpotatoes (*Ipomoea batatas* (L.) Lam.), beets (*Beta vulgaris* L.), turnips (*Brassica rapa* L.), parsnips (*Pastinaca sativa* L.), carrots (*Daucus carota* L.), and onions (*Allium* cepa L.). Dahlia roots were also included in the experiments. Besides the respiratory responses after cold storage the drift of the respiratory intensity with the age of the various vegetables was studied. Consideration was also given to the relation of the carbohydrates in the different vegetables to their respiratory intensity under different conditions, especially after cold storage.

REVIEW OF LITERATURE

As early as 1882 Müller-Thurgau (*12*) observed that potatoes which had been kept at 0° C. and then brought to 20° liberated more carbon dioxide than potatoes which had not been exposed to low tempera-

[1] Received for publication Mar. 30, 1936; issued December 1936.

[2] Reference is made by number (italic) to Literature Cited, p. 589.

ture. The evolution of carbon dioxide increased rapidly at first until the highest point was reached, and then sank gradually until it reached that of potatoes not previously kept at low temperature. It appears that Müller-Thurgau was concerned primarily with the effect on respiration of the sugar that accumulates in potatoes at low temperature rather than with the "change of temperature" effect.

Palladine (*16*) 17 years later exposed similar lots of tips of etiolated bean seedlings to medium, low, and high temperatures and then determined the evolution of carbon dioxide in each lot at a temperature of 18° to 22° C. The respiratory rate was stimulated by transferring the tips either from a low to a higher temperature or from a high to a lower temperature. He concluded that there is a stimulating effect on respiration caused by temperature changes, aside from the effect produced by the altered temperature.

In 1915 Appleman (*1*), using different varieties, verified Müller's earlier observation on potatoes and showed that McCormick potatoes which had been stored for 20 days at 3° C. and then brought to room temperature liberated in a unit of time 2.8 times as much CO_2 as a similar lot of potatoes that had been stored for the same length of time at room temperature. Kimbrough (*9*), working in the same laboratory, extended this study of the respiratory responses in potatoes with special reference to their storage and transportation. Potatoes were stored for different periods at constant low temperatures ranging from 0° to 10°, and the respiratory intensity in the potatoes after storage was determined at constant temperatures ranging from 10° to 30°. The practical implication of this work was to discover a temperature that would be suitable for potato storage and at the same time would reduce to a minimum the intensity and duration of the initial high respiration when the tubers were exposed to the higher temperatures.

Hasselbring and Hawkins (*7*) kept cured sweetpotatoes at a temperature of 6° to 9° C. from November 8 to December 9 and then measured the respiratory intensity of these roots at 30°. They found that the initial respiration of these chilled roots was much higher than that of roots which had not been subjected to the low temperature but, toward the end of the experiment, the respiration rates sank rapidly. Magness and Ballard (*10*) found no immediate acceleration of respiration in Bartlett pears after cold storage over fruit not held in cold storage other than the increase which would be accounted for by the slightly riper condition of the cold-storage fruit.

Magness and Burroughs (*11*) removed Winesap and Baldwin apples from storage temperatures to 18.2° C. and noted no differences that could be ascribed to the stimulation by low temperature in the rate of carbon dioxide evolution between samples from different storage temperatures. Burroughs (*4*) concluded that some stimulation of respiration occurred in the Wagener apples when they were held for 2 weeks at 0° C. and then removed to 18.5°, especially in the case of immaturely picked fruit. His data show a considerable variation in different experiments, but in general the respiration curves do not indicate the initial high respiration followed by a gradual fall to a lower level that is so characteristic of potatoes.

More recently Blackman and Parija (*3*) noted a special disturbance of initial rates of respiration in apples immediately after they were

heated from 2.5° to 22° C. The rising respiration did not simply mount up to the value for 22°, but in many cases clearly overshot that value and then fell back to it. Blackman and Parija speak of this as a minor feature that attracted attention in their study of respiration in apples.

Olney (*14*) determined the respiration rates in bananas at 20° C. He obtained a higher initial rate of respiration in refrigerated fruit than in fruit that had not been subjected to low temperature.

Hopkins (*8*) transferred potatoes from a cellar storage with an average temperature of about 10° C. to temperatures of 0° and 4.4° and measured the respiration rates at these low temperatures. The respiration rate at 0° passed through a maximum and then again approached its original value. Palladine also found a stimulation of respiration in etiolated bean seedlings immediately after they were transferred from a higher to a lower temperature. In all of the other papers here cited the initial rise in respiration rate was observed when the plant organs were transferred from a lower to a higher temperature.

EXPERIMENTAL PROCEDURE AND METHODS

GENERAL PROCEDURE

The parsnips, carrots, turnips, and beets used in these experiments were grown on a nearby commercial truck farm in fertile Sassafras loam soil. The vegetables were brought to the laboratory within an hour after harvesting and prepared immediately for the experiments. The tops were cut off one-fourth inch above the crown and all of the roots except the parsnips were washed thoroughly in running tap water. They were wiped as dry as possible with towels and then spread out on the laboratory table to dry for 2 hours before the lots were selected and weighed. The parsnips were brushed clean with a soft brush. The source and preparation of the other vegetables are described in connection with the experiments on these vegetables.

As a rule the respiration measurements at 22° C. were begun on the fresh vegetables the day they were harvested. At the same time other lots were stored at 2.5° for various periods and then transferred to 22° for respiration tests. At the beginning of all respiration measurements comparable lots were sampled for moisture and sugar determinations. The lots used for respiration were also sampled for moisture and sugar analysis at the conclusion of the respiration tests.

LOW-TEMPERATURE STORAGE

The vegetables were stored in a well-insulated box provided with a refrigeration unit which maintained in the box an average temperature of about 2.5° C. Since the fluctuations of temperature in the box were fairly regular over short intervals of time, the temperature of the vegetables in most cases was found to be fairly constant at 2.5° because of the lag in their response to small and regular fluctuations in the external temperature. The temperature of some of the small vegetables may have varied 1° to 2° at times during the storage period. Unless otherwise noted the various vegetables were placed in large moisture dishes with the lids so adjusted as to allow sufficient ventilation but to prevent excessive loss of water by evaporation during storage.

METHOD FOR MEASURING RESPIRATION

In view of the status of our knowledge of the respiratory process, it would seem that there is no entirely satisfactory method of experimentally measuring respiratory intensity in plants if we interpret this in terms of free energy. The method most commonly employed is to measure the amount of carbon dioxide liberated from a unit of plant tissue for a unit of time. There is no implication as to the metabolic source of the carbon dioxide, but it is assumed that it is the end product of a sequence of reactions in which energy is released. Although the amount of CO_2 liberated may in some cases be only an approximate measure of the total respiratory intensity, it is probably the most satisfactory measure for use in the present study. Some workers have determined the consumption of oxygen as a measure of the respiratory intensity, but since it is not definitely known just how and to what extent atmospheric oxygen enters into the total respiratory process, this method seems no more reliable than that of measuring the amount of CO_2 liberated. It is definitely known that under some circumstances oxygen may be consumed in metabolism other than respiration. Then, too, energy released by any anaerobic phase of the total respiratory process which liberates CO_2 would not necessarily be indicated by the oxygen consumption.

The method employed to measure the amount of carbon dioxide expired was similar to that described by Gore (*5*). The method and the apparatus used in this laboratory have been described in detail by Kimbrough (*9*).

In order to insure the removal of all carbon dioxide from the system and also to allow the vegetables to warm up to the temperature of the respiration chamber, carbon dioxide-free air was drawn over the material for 3 to 4 hours before the respiration measurements were begun. The expired carbon dioxide was measured at the end of each 24-hour period. All of the respiration determinations were made at a constant temperature of 22° C.

BASIS FOR CALCULATING RESPIRATION DATA

Having decided to use either the amount of carbon dioxide liberated or the amount of oxygen consumed as an approximate measure of the respiratory intensity in the living cells of a plant organ, one is still confronted with an important practical question. What basis shall be adopted for the calculation of experimental results? Shall a unit of fresh weight or a unit of dry weight be used? Because of the varying water content in plant tissues, a unit of dry weight would seem at first to be the most appropriate basis for the calculations, but this method does not take into account the possible influence of the water content on the respiratory intensity of the tissue.

Palladine (*15*) has proposed a method of calculation based upon the actual amount of protoplasm in the tissue. This method also leaves out of account the influence of other components of the tissue on the respiratory intensity, especially the water and carbohydrates. The variable amounts of such materials as cellulose and lignin in different plant tissues is probably the greatest source of error in comparing the respiratory intensity of different tissues and organs. Since we do not know the relative importance of the different com-

ponents of plant organs in determining their respiratory intensity as measured by the CO_2 liberated, it was thought best to use a unit fresh weight of the respiring system as the basis for calculating the experimental results of the present study.

ANALYTICAL METHODS

Sampling.—The vegetables were split longitudinally and either one-quarter or one-half of each was used in the composite sample. In some of the early experiments with parsnips the roots were ground in a small meat chopper and then in a mortar. In all of the other experiments the vegetables were ground to a fine pulp in a Nixtamal mill. The pulp was kept thoroughly mixed while the samples were being removed for analysis.

Moisture.—From 3 to 6 g of pulp were weighed in a tared watch glass and dried to constant weight in a slow current of dry air at 78° to 80° C. and at a pressure of 28 to 30 inches of mercury.

Sugars.—The amount of pulp used for the sugar determinations varied from 20 to 50 g, depending on the sugar content of the different vegetables. The samples of pulp were weighed into counterpoised 200-cc Kohlrausch flasks and covered immediately with 75 or 100 cc of boiling 95-percent alcohol. The flasks were then placed on a steam bath, and when the contents began to boil the alcohol was reduced to 70 percent by the addition of the calculated amount of hot water and the boiling continued for 30 minutes to extract the sugars. After cooling to 20° C. the flasks were filled to the mark with 95-percent alcohol and set aside until the sugar determinations could be made.

When the sugars were determined the extracts were filtered, and 125 to 150 cc of the filtrate was evaporated on a steam bath until all of the alcohol was removed. Water was added in small amounts to keep the volume of the solution at about 40 cc. The aqueous extracts were then transferred to 200-cc volumetric flasks and cleared with neutral lead acetate. The excess of lead was removed with anhydrous sodium carbonate. The flasks were then filled to the mark and suitable aliquots of the filtered solutions were used for the sugar determinations which were made by the method of Munson and Walker (*13*).

In making the final calculations no correction was made for the volume occupied by the undissolved solids of the pulp in the volumetric flasks. Hasselbring (*6*) made the correction for the volume occupied by 25 g of carrot pulp in 250 cc of solution and found that it changed the maximum percentage of sugar only slightly in the second decimal. In the present analysis the slight error would be constant throughout and it would not affect the comparative value of the results.

The percentages of total and reducing sugars on wet-weight basis at time of sampling were calculated as percentages of the wet weight of the vegetables at the time of harvest. These latter percentages are recorded in the tables as percentages of original wet weight and they serve to check apparent changes in percentages of sugar due to loss of water and carbon dioxide from the vegetables during storage.

RESULTS

POTATOES

Experiment 1

Willaman and Brown (*18*) concluded that the temporary flush of CO_2 output from apple twigs when they were raised from a lower to a higher temperature was caused by the lower solubility of the CO_2 at the higher temperature. They further concluded that in plant-respiration studies a careful distinction must be made between the production of CO_2 and its output.

Experimental data have been reported from this laboratory (*9*) that seem to prove rather conclusively that the initial high liberation of CO_2 from potatoes after a period of cold storage is due to actual respiration and not simply to the output of the excess CO_2 which was dissolved in the cold sap of the potato tissue. As further proof of the respiratory production of excess output of CO_2 by potatoes after cold storage an additional experiment is here reported.

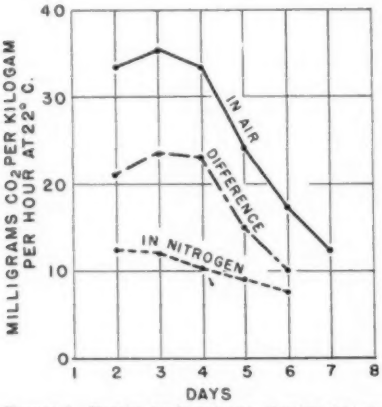

FIGURE 1.—Respiration in potatoes, showing that the initial high liberation of CO_2 after a period of cold storage is due largely to actual respiration.

Two comparable lots of potatoes were stored at a low temperature for the same length of time and then transferred to 22° C. for respiration tests. Carbon dioxide-free air was passed over one lot while pure nitrogen was passed at the same rate over the other lot. The carbon dioxide liberated was measured at 24-hour intervals. The results are shown in figure 1. It should be noted that most of the temporary excess of carbon dioxide liberated was from the lot in air. If the excess output of carbon dioxide were due entirely to the liberation of the carbon dioxide which was dissolved in the potato sap during the cold storage, then the lot in nitrogen should show the same temporary excess output of carbon dioxide as the lot in air, but this was not the case.

It would also be expected that most of the excess CO_2 that might be dissolved in the cold sap would be liberated as the potatoes warmed up to the respiration temperature and before the collection of the CO_2 was begun. This preliminary period was extended as long as 18 hours and the tubers were held under a high vacuum during this time, but still they showed the typical respiratory response after cold storage.

Experiment 2

Earlier respiration studies on potatoes (*9*) showed that when the tubers were stored for only 3 weeks at 2.5° C. and then transferred to 22°, the maximum initial respiration rate for these temperature conditions was obtained. The present study included an additional

experiment on potatoes which was designed to test the effect of repeating the low temperature storage for periods of 3 weeks alternating with periods of 3 weeks on respiration tests at 22°. Jersey Red Skins was the variety selected for his experiment. This variety was chosen because it showed promise of a desirable late variety for Maryland and its respiratory activity had never been determined.

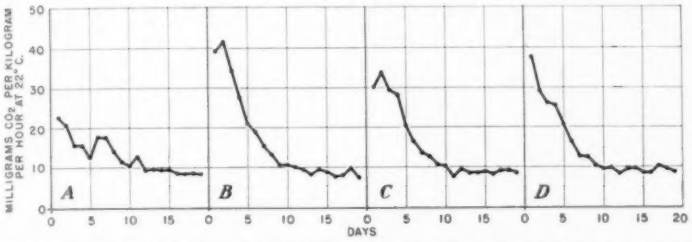

FIGURE 2.—Respiration in potatoes showing that the "change-of-temperature effect" can be reproduced at least three times in the same lot of potatoes without much change in the typical respiratory response: *A*, Respiration at 22° C., November 12 to December 6; *B*, stored at 2.5° December 6 to 29, respiration at 22° December 29 to January 19; *C*, stored at 2.5° January 19 to February 10, respiration at 22° February 10 to March 2; *D*, stored at 2.5° March 2 to 23, respiration at 22° March 23 to April 13.

The results of this experiment (fig. 2) show that the "change-of-temperature effect" can be reproduced in the same lot of potatoes at least three times over a period of 5 months without much change in the typical respiratory response.

SWEETPOTATOES

Sweetpotatoes were included in the vegetables used in this study for the purpose chiefly of comparing their respiratory responses with those of the other vegetables under similar conditions. Hasselbring and Hawkins (7) had previously conducted respiration studies with sweetpotatoes, but they were concerned primarily with the correlation between sugar content and respiration. At the same time they recognized an effect on respiration of the environmental conditions to which the sweetpotatoes had previously been subjected.

Sweetpotatoes of the Big Stem Jersey variety grown on the Maryland Experiment Station farm at College Park were dug October 25. After the roots had been brushed with a soft brush, nine lots of six roots each were selected, each lot averaging about 1,450 g in weight. Respiration measurements on one lot were begun the same day and continued until November 11. A similar lot was sampled for moisture and sugar analysis. Four lots were stored in open pans at 2.5° C. and three lots were stored at 29.3° to allow the curing processes to take place. The average of the daily mean temperatures for 22 days prior to the date when the sweetpotatoes were harvested was 13.7°.

The periods of storage at the different temperatures, together with respiration and analytical results, are shown in table 1, and in figure 3. The respiration data on the sweetpotatoes that were stored immediately after harvest at 2.5° C. for 24 days are not included. Although these sweetpotatoes were sound at the end of the cold-storage period, soft rot appeared in three of the roots during the respiration tests. All of the sweetpotatoes that had been allowed to cure before they

were placed in cold storage remained sound during the respiration measurements. The rate of respiration, which was high in the freshly harvested sweetpotatoes (fig. 3, *A*, *a*), declined rapidly at

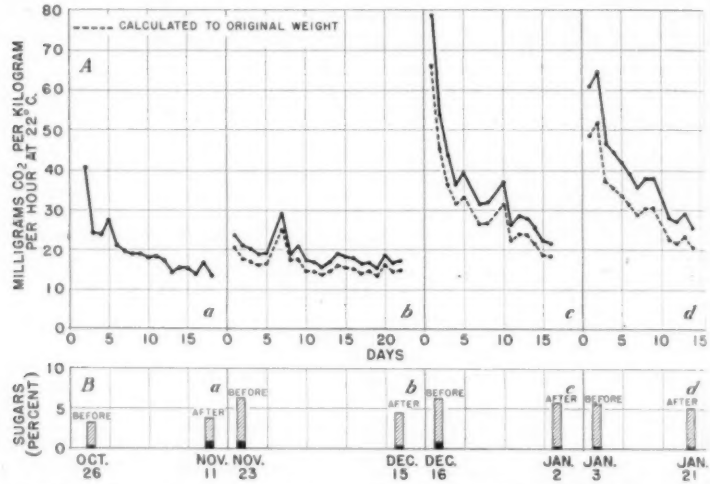

FIGURE 3.—*A*, Respiration in sweetpotatoes before and after storage at temperatures of 29.3° and 2.5° C.: *a*, Lot 1, at time of harvest; *b*, lot 2, after storage at 29.3° for 28 days; *c*, lot 3, after storage at 29.3° for 28 days, then at 2.5° for 23 days; *d*, lot 2, after the first respiration test stored again at 2.5° for 23 days. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugar.

first and then more slowly for about 12 days, when it began to fluctuate around a fairly constant average for the remainder of the 17-day respiration period.

TABLE 1.—*Moisture and sugars in freshly harvested and in cured sweetpotatoes before and after storage at 2.5° C.*

Storage period (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
FRESHLY HARVESTED, BEFORE AND AFTER STORAGE							
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
0	77.02	3.26	3.26	14.18	0.33	0.33	1.45
24	74.82	5.92	5.16	23.52	.61	.53	2.44
CURED, BEFORE AND AFTER STORAGE							
0	75.68	6.23	5.24	25.62	0.81	0.68	3.29
23	74.64	6.32	5.31	24.95	.81	.68	3.21

When the cured sweetpotatoes were stored at 2.5° C. and then transferred to 22° their initial respiration rate was nearly double that of the freshly harvested roots (fig. 3, *A*, *c*). The respiration rate declined rapidly for about 5 days and then irregularly and much more

slowly until it nearly reached, in 15 days, the rate in the cured sweetpotatoes before they were placed in cold storage. The sweetpotatoes that had been used for respiration tests after a period of curing (fig. 3, *A*, *d*) were also stored at 2.5° at the end of the respiration period. After 23 days at 2.5° the drift of the respiration curve at 22° was very similar to the one just described, although the initial rate was not as high.

The freshly harvested sweetpotatoes contained much less total and reducing sugars than the cured roots, but the initial respiration rate was much higher in the fresh uncured sweetpotatoes. During the respiration period of the freshly harvested roots the total sugars increased from 14.18 to 17.43 percent dry weight and the reducing sugars from 1.45 to 4.70 percent, but the rate of respiration declined to less than one-half of the initial rate. The percentage of both total and reducing sugars in the cured sweetpotatoes did not show any significant increase during 23 days' storage at 2.5° C. but the initial respiration rate in the roots after the cold-storage period was 3.5 times greater than the rate in cured roots before cold storage. The data from these experiments do not indicate a general correlation between either the total or reducing sugar content of the sweetpotato and its respiratory activity. The experiments of Hasselbring and Hawkins (7) likewise showed no correlation between total sugar content and respiration, but they claimed that their experiments indicated a correlation between reducing sugar content and respiration, although they admitted that such a correlation might be obscured by other factors.

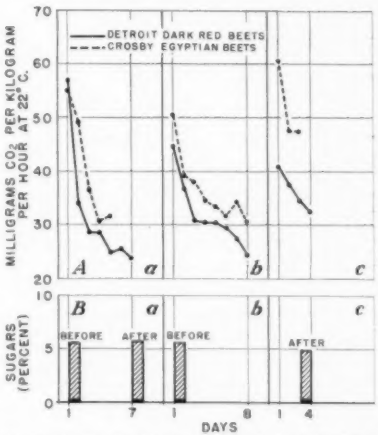

FIGURE 4.—*A*, Respiration in Detroit Dark Red and Crosby Egyptian beets before and after storage at 2.5° C.: *a*, Lot 1, at time of harvest; *b*, lot 2, after 26 days' storage; *c*, lot 2, after a second period, this time of 32 days, in cold storage. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugars.

BEETS

Two varieties of beets, Crosby Egyptian and Detroit Dark Red, were harvested October 23, and 4 lots of 10 beets each were selected from each variety. The average weight per lot was about 950 g for the Detroit Dark Red beets and about 830 g for the Crosby Egyptian. The experiments on the former variety were begun the day they were dug, and on the latter variety 2 days later.

At the end of the first cold-storage period, respiration measurements were continued for 8 days. The beets were then subjected to a second period of cold storage, and respiration tests made again for 4 days. The results of this experiment together with the storage periods are shown in table 2 and figure 4. The percentages of sugar in the two

varieties were so nearly the same that only those for the Detroit Dark Red are plotted.

TABLE 2.—*Moisture and sugars in Detroit Dark Red and Crosby Egyptian beets at time of harvest and after storage at 2.5° C.*

Storage at 2.5° C. (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
DETROIT DARK RED							
0	88.77	5.56	5.56	49.54	0.08	0.08	0.68
28	88.02	5.56	5.37	46.51	.10	.09	.82
CROSBY EGYPTIAN							
0	86.85	6.65	6.65	50.64	0.09	0.09	0.75
26	85.46	6.68	5.69	45.89	.11	.09	.81

The general drift of the respiration curve was the same in the two varieties, but the rate in the Crosby Egyptian beets was consistently higher at all times. The initial rate of respiration in the beets after the first period of cold storage was less than that in the freshly harvested beets, but the decline in rate with time was about the same. The initial rate of respiration after the second storage period at low temperature was considerably lower than the initial rate after the first period of cold storage, but it was nearly double the rate in the beets before the second storage, thus showing some effect on respiration rate of the sudden transfer of the beets from cold to warm temperature. No correlation of respiration rate with the sugar content could be detected in the beets. The much higher respiration in the Crosby Egyptian beets after the second period of cold was not due to a difference in sugar content, for the two varieties differed in this respect by only 0.05 percent.

TURNIPS

The turnips used for the experiments were the Purple Top White Globe variety, and were harvested October 23. Four lots were selected, 2 lots of 10 roots each and 2 lots of 8 roots each. Respiration measurements were started on one lot and another lot was sampled for analysis. The remaining two lots were stored at 2.5° C. for 28 days, after which one lot was sampled for analysis and the other was used for respiration tests for a period of 8 days. This lot was stored a second time at 2.5° for 32 days and then respiration measurements were made for 5 days. The results are given in table 3 and figure 5. The average of the mean daily temperature for a period of 3 weeks prior to the harvesting date was 14.3°.

The respiration rate in the freshly harvested turnips decreased rapidly during the first 2 days and then much more slowly but continuously until the end of the respiration measurements. The initial rate in the turnips that had been in cold storage was considerably less than the initial rate in the freshly harvested turnips, but after a temporary rise the decline in rate was about the same.

TABLE 3.—*Moisture and sugars in turnips at time of harvest and after storage at 2.5° C.*

Storage period (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
0	91.93	4.14	4.14	51.34	3.67	3.67	45.58
25	91.87	4.17	4.05	51.30	3.77	3.66	46.49

The respiration curve for the turnips after the second period of storage at low temperature was in general similar to that for the roots after the first cold-storage period. The initial rate was much higher than that in the roots just before the second cold storage. This would seem to indicate some change-of-temperature effect on the respiration in turnips.

In the fresh turnips the percentage of reducing sugars was much higher than the percentage of sucrose, which was relatively low in these vegetables. During storage of the roots at the low temperature there was a slight increase in reducing sugars, but there was no appreciable change in the total sugar during the 28 days of storage. It is apparent from the results of this experiment that there is no direct relationship between sugar content and intensity of respiration in turnips.

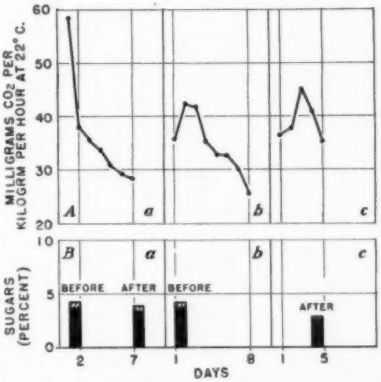

FIGURE 5.—*A*, Respiration in turnips before and after storage at 2.5° C.: *a*, Lot 1, at time of harvest; *b*, lot 2, after 28 days' storage; *c*, lot 2, after a second period, this time of 32 days, in cold storage. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugars.

PARSNIPS

Parsnips of the Hollow Crown variety were harvested November 11 and brought immediately to the laboratory. The tops were cut off about one-fourth inch above the crowns and the dirt removed from the roots by brushing with a soft brush. Ten lots of 8 roots each were selected for respiration determinations and 10 lots of 5 roots each were selected for sugar and moisture determinations. The lots used for respiration averaged about 700 g each, while those for sugar analysis averaged about 500 g each. One lot was placed immediately in the respiration chamber and another lot was sampled for sugar and moisture analysis. Eight lots were stored at 2.5° C. The roots for storage were placed on soil in 10-inch earthenware pots and covered with soil to a depth of about 1 inch. The pots were set in shallow pans in which was kept a little water to keep the soil damp. Under these conditions the roots actually increased slightly in weight

during the long storage periods and the percentage of water in the roots increased as shown in table 4.

TABLE 4.—*Moisture and sugars in parsnips at time of harvest and after soil storage at 2.5° C.*

Storage period (days)	Moisture	Total sugars		Reducing sugars	
		Wet weight	Dry weight	Wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*
0	79.20	4.30	20.67	0.20	1.11
21	78.75	7.00	32.91	.43	2.01
47	81.32	7.79	41.65	.40	2.14
92	83.10	7.17	42.50	.79	4.68
131	83.47	8.20	49.65	.65	3.95

Duplicate lots of roots were removed from storage for respiration tests and sugar and moisture determinations after 21, 47, 92, and 131 days in storage. The results on the soil-stored roots are shown in table 4 and in figure 6.

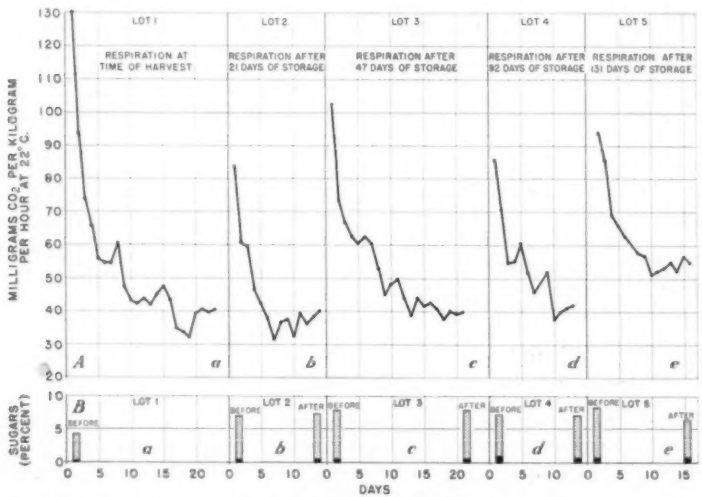

FIGURE 6.—*A*, Respiration in parsnips before and after storage in soil at 2.5° C.: *a*, Lot 1, at time of harvest; *b*, lot 2, after 21 days' storage; *c*, lot 3, after 47 days' storage; *d*, lot 4, after 92 days' storage; *e*, lot 5, after 131 days' storage. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugars.

The high rate of respiration in the parsnips immediately after harvest declined rapidly and steadily for the first 5 days and then much more slowly and irregularly for the remainder of the respiration period. The respiration in these roots seemed to be approaching a fluctuating rate around a fairly constant mean. Small shoots developed on some of the parsnips on the seventeenth day, but caused no apparent increase in the respiration rate. The field temperature

previous to harvesting may have had some effect on the initial rate of respiration in the fresh parsnips, as the average of the mean daily temperature for a period of 3 weeks just prior to harvesting the roots was 6.6° C. This was much lower than the temperature at which respiration determinations were made but was higher than the storage temperature.

The general drift of the respiration rate in the parsnips after the different periods of cold storage was very similar to that in the freshly harvested roots except that the initial rate was never as high and the final rate after the long storage periods was not as low as in the fresh parsnips.

There was no direct correlation between the sugar content of the parsnips and the rate of their respiration. The initial rate in the unstored lot was higher than that in any other lot, but the percentage of both reducing sugars and total sugar was lower than in any other lot. The lot stored for 21 days had about twice as much reducing sugar and more than a third more total sugar than the original fresh lot at the beginning of the respiration period.

Several lots of parsnips were also placed in small wire baskets and stored at the low temperature. The loss of water from these air-stored lots was so great that the respiration data are of interest only in showing the effect of water content on the respiratory activity of these roots. The relationship of both moisture and sugar content to respiration is shown in table 5. In this table only the respiration rate for the second day of each test is recorded. These figures seem just as satisfactory for comparing the respiratory activity in the different lots as the data for the entire period of the respiration test. After 47 days' storage the respiration rates per unit of dry weight were nearly the same in the soil- and air-stored lots, although the soil-stored lots contained 81.32 percent moisture while the air-stored lot contained only 57.45 percent. A further reduction of the percentage of moisture in the air-stored parsnips resulted in a corresponding decrease in respiration until it reached a very low value when the moisture content was reduced to 31.65 percent. After 21 days' storage the respiration rate of the air-stored parsnips was nearly twice as great as that of the soil-stored lot. This may or may not have been due to the fact that the sugar content after 21 days' storage was much higher in the air-stored lot and the water content was still optimum for respiratory activity.

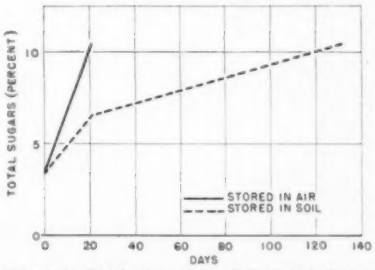

FIGURE 7.—Total sugar content (percentage) of parsnips when stored in air or in soil at 2.5° C.

It is a significant fact demonstrated by repeated experiments that the accumulation of sugar in parsnips at low temperature is much more rapid when the parsnips are freely exposed to air than when they are buried in the soil. (fig. 7.).

TABLE 5.—*Relation between respiration rate of parsnips and their moisture and sugar content after varying periods of storage at 2.5° C.*

[All percentages calculated to dry basis]

Storage period (days)	CO_2 per gram dry weight per hour		Moisture		Total sugars		Reducing sugars	
	Stored in soil	Stored in air	Stored in soil	Stored in air	Stored in soil	Stored in air	Stored in soil	Stored in air
	Mgm	*Mgm*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
21	6.83	12.75	78.75	74.75	32.91	51.71	2.01	7.10
47	9.41	8.89	81.32	57.45	41.65	48.35	2.14	5.88
92	9.98	3.82	83.10	39.88	42.50	46.65	4.68	2.75
131	13.63	.74	83.47	31.65	49.65	44.05	3.95	2.40

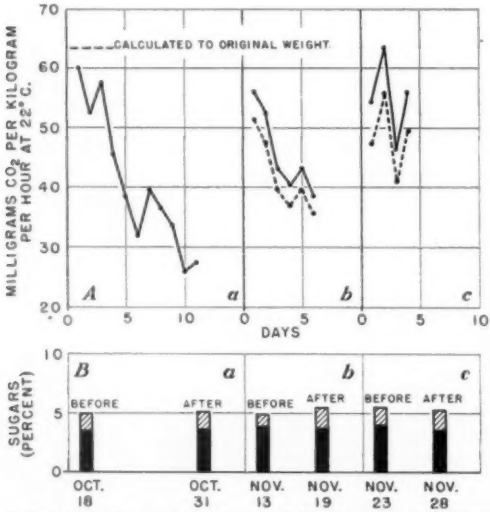

FIGURE 8.—*A*, Respiration in carrots used in experiment 1 before and after storage at 2.5° C.: *a*, Lot 1, at time of harvest; *b*, lot 2, after 26 days' storage; *c*, lot 3, after 36 days' storage. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugars.

CARROTS

EXPERIMENT 1

Danver Half-Long or closely related varieties were used for all of the experiments with carrots. The carrots for experiment 1 were harvested October 16, and stored in crates in a cool cellar until October 17, when lots of 15 carrots each were selected. Two lots were used for immediate respiration tests and one lot was sampled for sugar and moisture determinations. The remaining lots were stored at 2.5° C. At the end of 26 days and 36 days respectively, triplicate lots were removed for respiration tests and for moisture and sugar determinations. The results are shown in table 6 and in figure 8. The averages of the respiration rates of duplicate, lots are plotted.

TABLE 6.—*Moisture and sugars in freshly harvested carrots and after storage at 2.5° C.*

Storage period C (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
0	88.60	5.07	5.07	44.55	3.48	3.48	30.62
26	88.95	5.02	4.53	45.51	4.02	3.62	36.48
36	87.85	5.58	4.93	46.06	4.11	3.63	33.92

EXPERIMENT 2

Repeated respiration experiments with fresh carrots all showed a rapid fall in respiration rate with age of the roots. The initial respiration rate after a period of cold storage was never as high as the rate at time of harvest. This experiment was planned especially to decide whether the respiratory response in carrots after cold storage is a change-of-temperature effect or whether it merely represents the natural drift of the respiration rate due to senescence of the roots at the respiration temperature.

The carrots were harvested in the morning of November 12 and brought immediately to the laboratory and prepared for the experiment. Five lots of 10 carrots each were selected and each lot was treated as indicated in table 7.

TABLE 7.—*General plan of experiment 2, with carrots, and the loss in weight of each lot during each experimental period*

Lot no.	Period A, 2 weeks	Loss in weight	Period B, 3 weeks	Loss in weight	Period C, 2 weeks	Loss in weight
		Percent		*Percent*		*Percent*
1	Stored at 22° C	3.03	Stored at 2.5° C	1.75	Respiration 22° C	1.2
2	do	2.76	Stored at 22° C		do	1.8
3	do	2.81	do	4.7	do	1.4
4	Respiration 22° C	3.12	Stored at 2.5° C	3.1	do	1.4
5	Stored at 2.5° C	3.20	Respiration 22° C	2.3		

During period A moist air was passed through the chambers containing lots 1 and 2 at the same rate as for the respiration tests. Moist carbon dioxide-free air was passed through the chamber containing lot 3 in order to make the storage conditions of this lot exactly comparable to those of lot 4, on which respiration tests were being made during this period. Lots 1 and 2 were intended to check the effect of passing carbon dioxide-free air over lots 3 and 4 during period A. During period B all of the stored lots were placed in moist chambers with the covers slightly raised to allow ventilation.

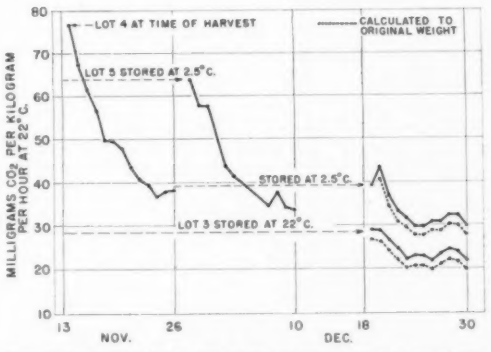

FIGURE 9.—Respiration in various lots of carrots, used in experiment 2 and shown in table 7, after storage at various temperatures for different periods.

The results of this experiment are shown in figure 9. The final respiration results for lots 1 and 2 were not plotted as they did not vary from those for lots 3 and 4 more than would be expected in different lots of carrots.

It seems quite evident from this experiment that the apparent change-of-temperature effect in carrots is simply the drift of respiration rate with senescence of the roots at the respiration temperature. The period of cold storage simply postponed the inevitable falling of the respiration rate with age.

Experiment 3

Another experiment was conducted with carrots of a crop grown the following year (1930) in order to test further the results and conclusions of experiment 2. The seed for this crop was sown in June of the very dry season of 1930. When the carrots were dug on October 21 the soil around the roots was almost a mass of dust, but nevertheless the moisture content of the carrots was 84.6 percent. Carrots from crops grown during the three previous seasons had had a moisture

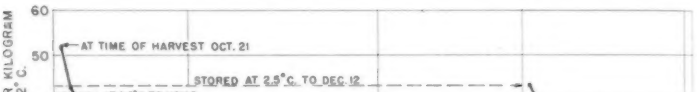

FIGURE 10.—Respiration in various lots of carrots used in experiment 3, after storage at temperatures of 22° and 2.5° C. for different periods to demonstrate the effect of senescence upon respiration.

content of 88.0, 87.3, and 88.6 percent, respectively. On the day the carrots were harvested five lots of eight roots each were selected for the experiment. The lots ranged in weight from 814.5 to 870.5 g, the average weight being 848 g. The respiration results as well as the storage treatments are shown in figure 10. The lot stored at 22° C. was placed in the respiration chamber with two glass tubes extended through the stopper to furnish ventilation. The lots stored at 2.5° were placed in moist chambers with the lids raised slightly. Under these conditions the roots lost little weight and remained sound for the period of the experiment. During the period of respiration measurements at 22° there was always more or less shoot growth but this growth did not appear to affect the respiration rate of the roots as measured by the carbon dioxide expired. Frequent removal of the shoots did not materially alter the respiration rate as compared with that of a lot from which the shoots were not removed.

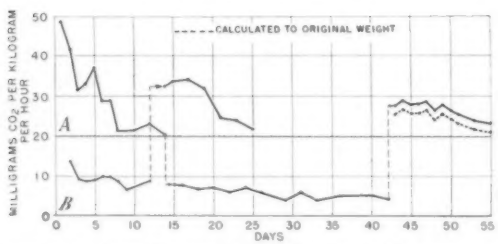

FIGURE 11.—Respiration in various lots of carrots used in experiment 4, stored at alternate temperatures of 22° (*A*) and 2.5° C. (*B*) for various periods.

Experiment 4

In experiment 4 the carrots were exposed to alternate temperatures of 2.5° and 22° C., but this experiment differed from previous ones in that respiration measurements were made at both the high and low temperature and were continued throughout the experiment. The roots therefore were exposed continuously to the experimental con-

ditions of the respiration tests. The experimental procedure and the respiration results are given in figure 11.

EXPERIMENT 5

Since carrots are commonly shipped and handled with the tops attached, respiration tests were made on roots and tops attached and on roots and tops separately. The experiment also served to test the effect of the wound stimulus when the tops are removed from the roots.

The carrots for this experiment were harvested November 30 and brought immediately to the laboratory. Lots of 10 roots each were selected. The tops were removed from one lot and the roots were placed at once in the respiration chamber. At the same time respiration tests were begun on another lot with tops attached. After 3 days the tops were removed from this lot and respiration tests continued on the roots and tops separately. The results are shown in table 8.

TABLE 8.—*Respiration in carrots with tops attached and in roots and tops separately*

Part and treatment	CO_2 per hour per kilo for 24-hour period indicated									
	1	2	3	4	5	6	7	8	9	10
	Mgm	*Mgm*	*Mgm*	*Mgm*	*Mgm*	*Mgm*	*Mgm*	*Mgm*	*Mgm*	*Mgm*
Roots	76.0	81.5	61.5	52.0	54.1	48.5	49.7	44.7	37.3	41.9
Roots and tops attached	103.4	125.8	104.8							
Roots after removal of tops				63.0	57.6	52.4	50.9	47.3	42.4	39.7
Tops				268.6	311.2	313.3				
Calculated values for root and top attached				107.1	110.9	108.3				

The respiration rate for roots and tops attached was more than double that of an equal weight of roots alone. The respiration rate of the detached tops was 4 to 6 times higher than that for an equal weight of roots.

Since the ratio of root weight to top weight was known, it was possible from the data on roots and tops separately to calculate the respiration rate for roots and tops attached.

A comparison of these calculated values with the actual data for respiration of roots and tops attached shows that there was only a very slight increase in respiration rate in the roots due to the wound stimulus from removal of the tops.

Platenius (17) states that the practice of storing bunched carrots even for a relatively short time cannot be recommended. He found that water moves at first from the roots into the leaves at a rapid rate, but later when the tops begin to die the water is drawn back into the roots. The high respiration in the tops (table 8) suggests the possible movement of solutes from roots to tops, as the carbohydrates in the tops would soon be exhausted by the high respiratory intensity at 22° C.

ONIONS

On October 17, onions of a yellow variety grown in Michigan were purchased on the market. On October 18, six lots of 10 onions each were selected. The average weight per lot was about 1 kg. Respira-

tion measurements were started the same day on one lot and a second lot was sampled for analysis. The other lots were stored at 2.5° C. in paper bags, which were perforated in a few places to insure ventilation. Duplicate lots were removed from storage at the end of 23 and 49 days, respectively, for analysis and respiration determinations. The results are given in table 9 and in figure 12.

TABLE 9.—*Moisture and sugars in onions before and after storage at 2.5° C.*

Storage period (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
0	90.50	6.42	6.42	67.57	3.26	3.26	34.32
23	90.44	6.40	6.30	66.91	3.61	3.55	37.79
49	90.85	5.87	5.66	64.22	3.79	3.85	41.43

The respiration rates of onions were relatively low in all cases, but the initial rates in the fresh onions and in those after periods of cold storage were higher than the final rates attained for the period of the tests. The initial rates after storage were practically the same as the initial rate in fresh onions, but the final rates were lower than the final rate of the fresh onions.

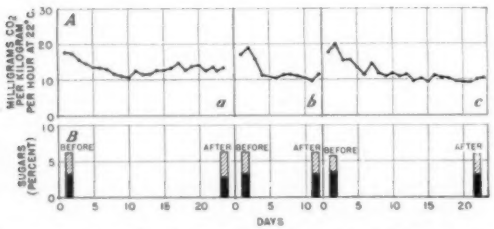

FIGURE 12.—*A*, Respiration in onions before and after storage at 2.5° C.: *a*, Lot 1, before storage; *b*, lot 2, after 23 days' storage; *c*, lot 3, after 49 days' storage. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugar and the shaded portions reducing sugars.

The total sugar content of these onions was relatively high, being 67.5 percent of the dry weight. The reducing sugars accounted for approximately one-half of the total sugars. During cold storage there was no accumulation of total sugar in the onions, but the reducing sugars seemed to increase slightly with the cold-storage period. From the data obtained there appears to be no general correlation between sugar content and respiration in onions.

DAHLIA ROOTS

Dahlia roots were included in this study because they store reserve carbohydrates in the form of inulin which upon hydrolysis gives rise to levulose. The roots are also stored for long periods.

The dahlia roots used in this experiment had been stored in a cool cellar from the time of harvest until February 29, when they were brought to the laboratory and prepared for the experiment. The roots were cut from the stalks, washed in running tap water, wiped with towels, and allowed to dry on the laboratory table for 2½ hours. Four lots of 11 roots each were then selected. The average weight per lot was about 760 g. Respiration determinations were started immediately on one lot, and one lot was sampled for analysis. The other two lots were placed in ventilated moist chambers and stored at 2.5° C. for 23 days. At the end of this period one lot was used for respiration tests and the other for analysis. The lot used for the respiration measurements for a period of 8 days was again stored at 2.5° for 21 days, after which respiration determinations were made again for 11 days. The results are given in table 10 and figure 13.

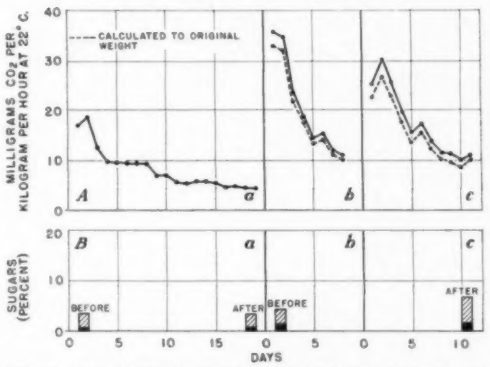

FIGURE 13.—*A*, Respiration in dahlia roots before and after storage at 2.5° C.: *a*, lot 1, before storage; *b*, lot 2, after 23 days' storage; *c*, lot 2, after a second period of 23 days' storage at 2.5° C. *B*, Sugar content of the different lots before and after the respiration tests. The height of the columns indicates total sugars and the shaded portions reducing sugars.

TABLE 10.—*Moisture and sugars in dahlia roots before and after storage at 2.5° C.*

Storage period (days)	Moisture	Total sugars			Reducing sugars		
		Wet weight	Original wet weight	Dry weight	Wet weight	Original wet weight	Dry weight
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
0	82.11	3.47	3.47	19.40	0.67	0.67	3.75
23	81.23	4.16	3.80	22.20	1.39	1.27	7.40

The type of respiratory response in the dahlia roots after a period of cold storage was very similar to that in potatoes and sweetpotatoes, although the rate in the latter was much higher. Both the total and reducing sugar increased during the cold-storage period.

COMPARISON OF RESPIRATION RATES OF DIFFERENT VEGETABLES IMMEDIATELY AFTER HARVEST

A comparison of the respiration rates of the different vegetables immediately after harvest is presented in figure 14. This comparison is based upon equal weights of the fresh vegetables and also upon equal weights of dry matter. On the fresh-weight basis the vegetables ranked in the following descending order in respect to initial respiratory intensity: Parsnips, carrots, beets, turnips, sweetpotatoes, potatoes, and onions. On the dry-weight basis the vegetables ranked in the following descending order: Turnips, parsnips, carrots, beets, onions, and sweetpotatoes. The shifting in the order of the vegetables when the respiration rate is based upon wet and dry weight cannot be explained entirely by the percentage of moisture in the various vegetables. The time rate of respiration in the dahlia roots was very similar to that in potatoes.

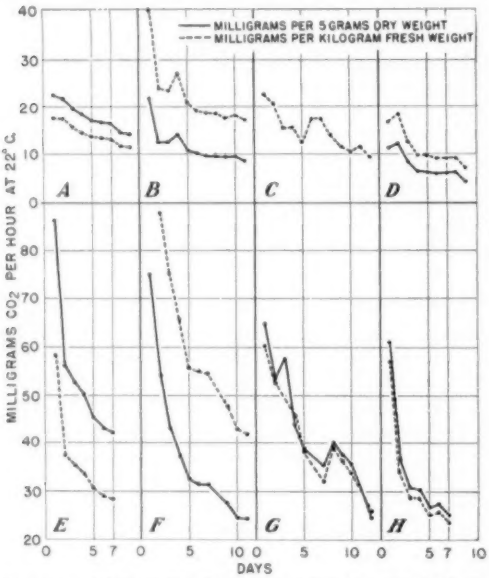

FIGURE 14.—Comparative respiration rates of various vegetables, and of dahlia roots, immediately after harvest: *A*, Onion; *B*, sweetpotato; *C*, potato; *D*, dahlia root; *E*, turnip; *F*, parsnip; G, carrot; *H*, beet.

RELATION OF SUGAR CONTENT TO RESPIRATION

The vegetables that accumulated sugar at low temperature were the ones in which the respiration rates at 22° C. were most altered by previous cold storage. Müller-Thurgau (*12*) observed that the rate of respiration in potatoes when they were transferred from a low to a higher temperature gradually declined as the sugar in the tubers was reconverted into starch. He therefore concluded that the sugar which accumulated in the tubers during cold storage was responsible for the initial increase in respiratory intensity when they were transferred to a higher temperature.

The experiments here reported failed to show any direct relationship between sugar content and respiration rate in the vegetables studied. The following typical experiments afford further evidence that Müller-Thurgau was mistaken in his conclusions regarding the relation of sugar content to respiration in potatoes.

EXPERIMENT 1

Potatoes that had been at low temperature long enough to accumulate 1.5 percent of sugar were transferred to 30° C. for respiration

measurements. The drift of respiration rate was typical, being high at first and then declining gradually to a much lower rate. During the period of the most rapid decline in the respiration rate the percentage of both total and reducing sugars in the tubers was actually increasing. It has long been known that the accumulated sugar in potatoes during cold storage is reconverted into starch when the potatoes are transferred to a warmer temperature. Therefore it was surprising to find the sugar continuing to increase for a time at 30° (fig. 15). However, further studies showed that in potatoes the shifting equilibrium between sugar and starch tends to attain stability with widely different percentages of sugar characteristic of different temperature ranges. In the variety of potatoes used in this experiment the equilibrium at 30° was attained when the total sugar in the potatoes was about 2.5 percent. Since the tubers contained only about 1.5 percent total sugar when removed from the low temperature the sugar continued to increase until equilibrium characteristic of 30° was established. In another experiment the tubers contained 4.3 percent sugar when transferred from low temperature to 30°, so the sugar in these potatoes decreased until it was reduced to 2.5 percent characteristic of equilibrium at 30°. Since the carbohydrate equilibrium is attained at 20° to 25° when the total sugar content is a fraction of 1 percent the usual observation is a marked decrease in the sugar content of cold-storage potatoes, after they are removed to a warmer temperature. It was therefore natural to infer that these carbohydrate transformations in potatoes with temperature changes were the cause of the characteristic respiratory response with the same temperature changes.

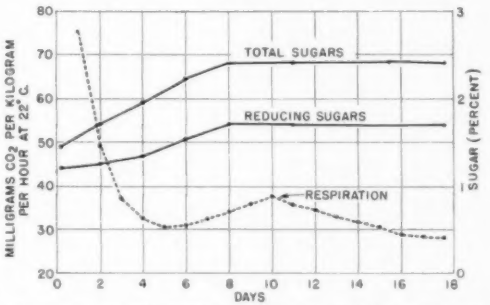

FIGURE 15.—Relation between total sugars, reducing sugars, and respiration rate of potatoes held at 30° C.

EXPERIMENT 2

McCormick potatoes that had been stored for a time at 29° C. were found to contain 2.05 percent total sugar. A lot of these potatoes was used for respiration measurements. After the respiration tests the tubers were placed at room temperature for 7 days and then stored at 0° for 12 days. After the period of cold storage they contained 2.18 percent total sugar. Respiration measurements were again made on a sample of this lot of tubers. The results, shown in table 11, indicate that the initial respiration rate in the cold-storage potatoes at the time that they were moved to 29° was more than two and one-half times the initial respiration at the same temperature in the tubers after storage at 29°, although the total sugar content was practically the same in both lots of potatoes.

TABLE 11.—*Relation between respiration rate of potatoes and their sugar content after storage at 29° and 0° C.*

Previous storage temperature (° C.)	Total sugar	CO_2 per kilogram per hour at 20° C.	
		First 24 hours	Second 24 hours
	Percent	*Milligram*	*Milligram*
29	2.05	29.3	24.3
0	2.18	77.8	29.7

DISCUSSION

The respiratory intensity in the different vegetables immediately after harvest varied greatly. The time rate of respiration in potatoes, sweetpotatoes, parsnips, onions, and dahlia roots declined rapidly at first and then more slowly until it began to fluctuate around a fairly constant average rate for the remainder of the respiration period. In the case of carrots, beets, and turnips, the respiration rate declined steadily until the end of the respiration period. Benoy (*2*) subjected 10 different kinds of green vegetables of edible maturity to respiration tests during the first 30 hours after they were harvested. Two of the vegetables, carrots and beets, were used in the present study, and the general trend of respiration in these vegetables immediately after harvest was much the same as that found by Benoy, except that in her experiments respiration seemed to approach a constant rate after the first 12 hours. This may have been due to the fact that her experiments lasted only 30 hours.

Since the initial rate of respiration in the freshly harvested vegetables declined during the period of the respiration tests it was difficult to decide whether the respiratory response after cold storage was due to a sudden change of temperature or whether it merely represented the natural drift of the respiration rate due to the senescence of the vegetables at the respiration temperature. Experiments especially planned to decide this point showed that the high initial respiration rate in potatoes, sweetpotatoes, and dahlia roots when they are suddenly changed from a lower to a higher temperature is a definite change-of-temperature effect.

Potatoes were subjected to 3 weeks' storage at 2.5° C. and then to 3 weeks' respiration tests at 22°. This procedure could be repeated three times on the same lot of potatoes over a period of 5 months without any significant change in the typical respiratory response after each succeeding period of cold storage. Potatoes responded only to a change from a lower to a higher temperature and not to a change from a higher to a lower temperature.

There was also a distinct change-of-temperature effect on the respiration rate of beets and turnips, but it was very much less pronounced.

The respiration rate in carrots when they were transferred from a low to a higher temperature declined rapidly and steadily. This might easily have been interpreted as a change-of-temperature effect

but it proved to be the natural drift of the respiration rate during senescence of the roots. The senescence processes were merely deferred during the periods of cold storage.

The drift of the respiration rate in parsnips after varying periods of cold storage was very similar to that in the freshly harvested roots at the same temperature, but the initial rate after cold storage was never as high as the initial rate in the fresh parsnips. The experiments with parsnips do not permit definite conclusions regarding the change-of-temperature effect on the respiration rate in these roots.

The respiration rate in the onions was low at all times and previous cold storage had little or no effect on the respiration rate at the constant higher temperature.

SUMMARY

A comparative study was made of the change-of-temperature effect on the time rate of respiration in a number of fleshy vegetables. The respiration measurements were made at 22° C. after the vegetables had been stored for varying periods at 2.5°. The drift of the time rate of respiration with age of the various vegetables was included in the study. Consideration was also given to the relation of moisture and carbohydrates in the different vegetables to their respiration intensity.

The choise of a method for measuring respiration intensity and the proper basis for calculating respiration data are discussed.

Further experimental proof of the respiratory production of the excess output of carbon dioxide by potatoes after cold storage is reported.

The effect of previous cold storage on the initial respiration rate at higher market temperatures varied with the different vegetables, being very pronounced in potatoes but not detectable in carrots. The vegetables in which the percentage of starch was relatively high and in which there was a rapid shifting of the carbohydrate equilibrium with temperature changes were the ones that showed the greatest increase in intial respiratory rate when they were transferred from a low to a higher temperature. The actual respiratory intensity in these vegetables, however, was lower than in most of the other vegetables studied.

The sugar that accumulates in starchy vegetables at low temperature is not responsible for the initial high respiration in these vegetables when they are moved to higher temperatures. In potatoes the shifting equilibrium between sugar and starch tends to attain stable positions with widely different percentages of sugar characteristic of different temperature ranges. Potato tubers accumulated sugar for a time at 30° C. as well as at temperatures just above freezing, but the sugar that accumulated in the tubers at the high temperature did not affect the respiration rate.

There was no direct correlation between the content of either total or reducing sugars and the respiration rate in the vegetables studied.

LITERATURE CITED

(1) Appleman, C. O.
1915. relation of catalase and oxidase to respiration in plants. Md. Agr. Expt. Sta. Bull. 191, pp. 1–16, illus.

(2) Benoy, M. P.
1929. the respiration factor in the deterioration of fresh vegetables at room temperature. Jour. Agr. Research 39: 75–80, illus.

(3) Blackman, F. F., and Parija, P.
1928. analytic studies in plant respiration. I.—the respiration of a population of senescent ripening apples. Roy. Soc. [London], Proc. (B) 103: 412–445, illus.

(4) Burroughs, A. M.
[1923]. . . . studies in apple storage . . . *In* Storage Investigations, 1921–1922. Marble Lab., Inc. Rept. 3: 101–138, illus. Canton, Pa.

(5) Gore, H. C.
1911. studies on fruit respiration . . . U. S. Dept. Agr., Bur. Chem. Bull. 142, 40 pp., illus.

(6) Hasselbring, H.
1927. carbohydrate transformations in carrots during storage. Plant Physiol. 2: 225–243.

(7) ——— and Hawkins, L. A.
1915. respiration experiments with sweet potatoes. Jour. Agr. Research 5: 509–517.

(8) Hopkins, E. F.
1924. relation of low temperatures to respiration and carbohydrate changes in potato tubers. Bot. Gaz. 78: 311–325, illus.

(9) Kimbrough, W. D.
1925. a study of respiration in potatoes with special reference to storage and transportation. Md. Agr. Expt. Sta. Bull. 276, pp. [51]–72, illus.

(10) Magness, J. R., and Ballard, W. S.
1926. the respiration of bartlett pears. Jour. Agr. Research 32: 801–832, illus.

(11) ——— and Burroughs, A. M.
[1923]. studies in apple storage. *In* Storage Investigations, 1921–1922. Marble Lab., Inc. Rept. 2: 17–98, illus. Canton, Pa.

(12) Müller-Thurgau, H.
1882. ueber zuckeranhäufung in pflanzentheilen in folge niederer temperatur. ein beitrag zur kenntniss des stoffwechsels der pflanzen. Landw. Jahrb. 11: [751]–828, illus.

(13) Munson, L. S., and Walker, P. H.
1906. the unification of reducing sugar methods. Jour. Amer. Chem. Soc. 28: [663]–686.

(14) Olney, A. J.
1926. temperature and respiration of ripening bananas. Bot. Gaz. 82: 415–426, illus.

(15) Palladine, W.
1896. recherches sur la corrélation entre la respiration des plantes et les substances azotées actives. Rev. Gén. Bot. 8: [225]–248.

(16) ———
1899. influence des changements de tempërature sur la respiration des plantes. Rev. Gén. Bot. 11: [241]–257.

(17) Platenius, H.
1934. physiological and chemical changes in carrots during growth and storage. N. Y. (Cornell) Agr. Expt. Sta. Mem. 161, 18 pp., illus.

(18) Willaman, J. J., and Brown, W. R.
1930. carbon dioxide dissolved in plant sap and its effect on respiration measurements. Plant Physiol. 5: 535–542, illus.

THE pH GRADIENT EXTENDING FROM THE PHLOEM INTO THE PARENCHYMA OF THE SUGAR BEET AND ITS RELATION TO THE FEEDING BEHAVIOR OF EUTETTIX TENELLUS [1]

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INTRODUCTION

Certain preliminary studies pertaining to the chemical nature of resistance to the curly top disease indicated the possibility that differences in hydrogen-ion concentration might be involved. The pH value of the expressed juice of a number of species of plants showing varying degrees of resistance to curly top was determined with the aid of the quinhydrone electrode. The freshly extracted juice from *Chenopodium murale* L., a very resistant species, consistently showed a distinctly alkaline reaction (pH 7.3 to 8).[2] By means of the same electrode it was found that juice extracted from sugar beet leaves was always acid (pH 5.8 to 6.5).

From these observations the suggestion arose that if by subjecting *Chenopodium murale* to a high concentration of carbon dioxide the cell sap of the plant could be made acid in reaction the plant might then become susceptible to curly top. Upon treatment of the plant with carbon dioxide, however, the reaction of the extracted juice of *C. murale* became more alkaline rather than more acid as was expected. Sugar beets were then treated with carbon dioxide and found to react in a similar way. The chemical studies and the probable reactions involved have been presented (*6*).[3]

Although the reaction in the cell sap resulting from carbon dioxide treatment was contrary to that expected, nevertheless the possible influence of this reaction on curly top resistance in sugar beets was investigated.

CARBON DIOXIDE TREATMENT OF SUGAR BEET SEEDLINGS

TREATMENT BEFORE AND DURING INOCULATION

In certain experiments beet seedlings were inoculated during their exposure to carbon dioxide. For these studies, a special type of chamber was required in order that the seedlings might be exposed to carbon dioxide and at the same time be inoculated by means of viruliferous beet leafhoppers, *Eutettix tenellus* (Baker), without subjecting the insects to the gas.

A tight box (fig. 1), with one side of glass, was constructed for the purpose of treating beet plants with carbon dioxide before and during inoculation. A beveled slit 1 inch wide at the top was cut the length of the lid. A strip of rubber sheeting was glued over the opening and

[1] Received for publication Mar. 9, 1936; issued December 1936.

[2] Determinations made at a later date revealed that these values, although constant, were high and misleading. When the juice expressed from *Chenopodium murale* was allowed to stand in the refrigerator for 3 days before pH determinations were made, true values (*6*)[3] near pH 6.4 were obtained.

[3] Reference is made by number (italic) to Literature Cited, p. 593.

securely held in place by thin strips of wood nailed on both edges. A slit the same length as the opening in the lid was then cut in the rubber. The edges of the rubber slightly overlapped, thus making an almost airtight seal. A large number of seedlings in a crystallizing dish were placed in the box and the tight-fitting lid clamped on. A definite concentration of carbon dioxide was built up in the box and maintained for a given period by admitting air and carbon dioxide at a constant rate. The ratio of air to carbon dioxide, forced through the chamber, was controlled by needle valves, with the aid of flow meters that had been previously calibrated.

The plants were inoculated by drawing the tip of one cotyledon of each plant through the slit in the rubber with a pair of rubber-tipped forceps and placing on it a cage containing four viruliferous leafhoppers. The opening in the lid was long enough to provide space for 20 plants to be inoculated at the same time. The insects were caged on the tip of the cotyledon and allowed to feed for one-half hour. After the feeding period the cages were removed, together with those portions of the cotyledons that were outside the gas chamber during the feeding. The plants were allowed to fall to the bottom of the chamber, where they remained in an atmosphere saturated with

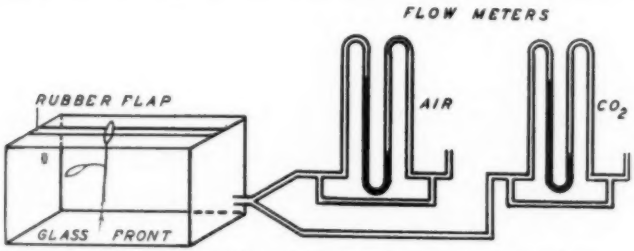

FIGURE 1.—Inoculating chamber and flow meters for controlling the gas mixture.

moisture until all the plants had been inoculated. After all the plants in the box had been inoculated, they were removed and planted in sand. Control plants were treated in the same manner, except that they did not receive the carbon dioxide treatment.

TABLE 1.—*Percentage of curly top occurring on normal beet seedlings and on those exposed to high concentrations of carbon dioxide immediately before and during inoculation*

Experiment no.	Plants		Concentration of CO_2 applied	Length of CO_2 treatment	Plants infected	
	Check	Treated with CO_2			Check	Treated with CO_2
	Number	*Number*	*Percent*	*Hours*	*Percent*	*Percent*
1	64	63	38	4	75	19
4	32	80	28	2	47	9
5	131	127	20	2	12	0
6	108	100	25	2	31	9
8	117	100	60	3	36	10
9	36	20	40	3	17	0

The data in table 1 are typical of the results obtained in a large number of experiments. It would appear from this table that the

plants subjected to carbon dioxide were rendered highly resistant to curly top. The ratio of the average percentage of infection in the controls to the average percentage of infection in the plants treated with carbon dioxide is 4.7 to 1. In some experiments the leafhoppers were allowed to feed on plants treated with carbon dioxide in darkness, while in others the plants were in the light during feeding. The percentage of infection resulting from these tests evidently was not affected by light.

Among the different experiments a wide variation was found in the degree of resistance to infection exhibited by the plants treated with carbon dioxide. Several factors probably contributed to bring about such a variation. In the first place, the concentration of carbon dioxide applied and the length of treatment were different for each experiment. It is possible also that the nutritional condition of the plants may have differed sufficiently to contribute to the observed variation.

It was found (*6*) that plants well nourished, especially with abundant nitrogen, responded to carbon dioxide treatment more quickly and to a much greater degree than slow-growing nitrogen-starved plants. The increase in pH values due to carbon dioxide treatment was much greater in the well-nourished plants. The nitrogen changes were also greater.

TREATMENT IMMEDIATELY AFTER INOCULATION

If the biochemical changes induced in the beet plant by carbon dioxide treatment before inoculation inactivate the virus or render it incapable of multiplication, it would appear logical to expect that treatment of the plant with carbon dioxide immediately following inoculation would reduce the amount of infection.

Experiments were therefore conducted to determine whether the virus could be inactivated by treating plants with carbon dioxide immediately following inoculation. Viruliferous leafhoppers were placed on seedlings for one-half hour, four leafhoppers per plant. One lot of the plants was immediately treated with carbon dioxide, while the untreated remainder served as controls. The plants were then transplanted to sand and the amount of infection was noted. It is evident from table 2 that the carbon dioxide treatment immediately following inoculation did not influence the percentage of plants infected and therefore that the treatment did not cause the plants to become resistant to the curly top virus. The average incubation period and also the severity of the disease on the plants treated with carbon dioxide were found to be the same as those observed on the control plants.

TABLE 2.—*Percentage of curly top occurring on normal beet seedlings and on those exposed to high concentrations of carbon dioxide immediately after inoculation*

Experiment no.	Plants		Concentration of CO_2 applied	Length of CO_2 treatment	Plants infected	
	Check	Treated with CO_2			Check	Treated with CO_2
	Number	*Number*	*Percent*	*Hours*	*Percent*	*Percent*
1	90	78	35	5½	92	90
2	131	29	20	4	10	14
3	98	80	20	32	61	54

From the data shown it must be concluded that the biochemical changes that took place in the plant as a result of the carbon dioxide treatment after the plant had been inoculated were not important factors in preventing the establishment of the virus.

The question then arose as to whether the reduction in infection resulting from the treatment of the plants with carbon dioxide prior to and during inoculation (table 1) might have been due to a disturbance of the leafhopper's normal way of feeding.

In view of the magnitude of certain of the biochemical changes (*6*) brought about by the cells of the sugar beet when subjected to a high concentration of carbon dioxide, it seems logical that these or other chemical reactions that may take place may have a pronounced effect on the leafhopper. For example, the hydrogen-ion concentration of the juice of beet leaves treated with carbon dioxide was decreased in some cases to one-hundredth of its normal value; the ammonia nitrogen was found to more than double, while at the same time the amide nitrogen was reduced to one-half its normal value.

This theory is further supported by the fact that the leafhopper normally feeds in the phloem (*1*), and by the evidence presented by Bennett (*1*) which indicates that the insect must introduce the virus into the phloem in order to insure infection.

The extent to which these biochemical changes interfere with the leafhopper's normal way of feeding may be determined by observing in which tissue the leafhopper's mouth parts terminate.

FEEDING BEHAVIOR OF EUTETTIX TENELLUS

TISSUE FROM WHICH LEAFHOPPERS FEED WHEN CAGED ON NORMAL AND ON CARBON DIOXIDE-TREATED PETIOLES

Plants in approximately the six-leaf stage, having petioles 3 to 4 inches in length, were treated with approximately 50-percent carbon dioxide for 2 hours. The plants were then removed from the chamber, and leafhoppers were caged on the petioles for 20 minutes. After this treatment, the petioles from the carbon dioxide-treated and control plants were hand-sectioned and permitted to stand overnight in water. The sheaths left by the insects while feeding take on a yellow color if allowed to stand in water (*1*), so there was no difficulty in determining the tissue in which the leafhoppers had fed. The trails found, on microscopic examination, were classified according to the tissue in which they terminated and according to their position with respect to the phloem. Detailed results obtained from four experiments are presented in table 3.

When leafhoppers were forced to feed on the petioles of plants treated with carbon dioxide it was obvious that they lost their sense of direction and that their reaching the phloem was a mere matter of chance. From the data it would appear that the insects on the treated petioles were even unaware of their nearness to the phloem, as not one changed the direction of its mouth parts in an effort to reach this tissue. In contrast to this, in three of the four experiments a significant number of leafhoppers feeding on the controls changed the direction of their mouth parts in order to reach the phloem. Of the 95 feeding trails observed in the controls 7 were found to curve in the parenchyma tissue and, as a result, to terminate in the phloem.

TABLE 3.—*Disturbance of the normal feeding habit of the leafhopper when forced to feed on sugar-beet petioles treated with carbon dioxide*

Experiment no. and treatment	Punctures	Total punctures which—					
		Hit phloem direct	Curved in parenchyma tissue to hit phloem	Reached phloem	Were deep enough but missed phloem	Were in direction to hit phloem but not deep enough	Were not in direction to hit phloem and not deep enough
	Number	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
1—Checks	23	47.83	8.70	56.53	0	17.39	26.09
CO_2 treated	22	4.54	0	4.54	9.09	36.36	50.00
2—Checks	21	57.14	0	57.14	9.52	19.05	14.29
CO_2 treated	13	15.38	0	15.38	7.69	30.77	46.15
3—Checks	28	46.43	14.28	60.71	3.57	28.57	7.14
CO_2 treated	33	12.12	0	12.12	21.21	42.42	24.24
4—Checks	23	43.48	4.35	47.83	0	39.13	13.04
CO_2 treated	31	16.12	0	16.12	9.68	16.12	58.06
Total—Checks	95	48.42	7.37	55.79	3.16	26.32	14.74
CO_2 treated	99	12.12	0	12.12	13.13	31.31	43.43

In view of the absence of probing, it appeared that the leafhoppers feeding on the petioles treated with carbon dioxide were satisfied as long as their mouth parts were inserted anywhere just beneath the epidermis. Of the total trails, 43 percent were not in the proper direction nor were they deep enough to reach the phloem. Only 31 percent of the trails were in the right direction to reach the phloem, but these were not deep enough. That is, approximately 74 percent of all the trails found in the treated petioles were not deep enough to reach the phloem, regardless of the direction in which they pointed.

In summing up the four experiments, it is seen that a total of 55.8 percent of the trails found in the untreated petioles terminated in the phloem, as compared to only 12.1 percent found in the phloem of the treated petioles. The ratio is 4.6 to 1. The striking agreement between this ratio and the ratio (4.7 to 1, table 1) of infection in normal plants to that in plants treated with carbon dioxide is further evidence to support that presented by Bennett (*1*), which indicates that the virus must be deposited in the phloem in order to insure infection.

FEEDING BEHAVIOR OF LEAFHOPPERS ON AN ARTIFICIAL FOOD ADJUSTED TO TWO DIFFERENT pH VALUES

Inasmuch as leafhoppers normally feed from the phloem, experiments were conducted to determine whether they prefer food that is alkaline rather than acid in reaction.

An artificial food consisting of 8 percent of sucrose in tap water was prepared. Sufficient sulphuric acid and a small amount of disodium phosphate were added to one-half of the liquid to stabilize the hydrogen-ion concentration at pH 5.0. The remainder of the liquid received the same amount of phosphate and sufficient sodium hydroxide to maintain the hydrogen-ion concentration at pH 8.5. To the under side of the membrane of a large cage, similar to that shown by Bennett (*2*) but 8.5 cm in diameter, were added large drops of the acid and of the alkaline food in three alternate rows of 6 drops each, making a total of 36 drops. Twenty-five leafhoppers were placed in the cage. The

numbers of leafhoppers sitting over the acid and over the alkaline drops were noted at the end of each 5-minute interval for 50 minutes.

Two similar experiments were conducted, in which the membranes of two cages, 8.5 and 4.0 cm in diameter, respectively, were marked off in quadrants. Sufficient agar-agar was added to the artificial food to produce a solid medium when poured in thin layers on plate glass. Sections of the solidified food were cut the size of the quadrants and pressed on the membranes, the acid food being placed on opposite quadrants. Twenty-five leafhoppers were placed in each cage and left unmolested. The number of leafhoppers sitting over the acid and over the alkaline food were counted at the end of each 5-minute interval for 40 minutes. The data obtained in the three experiments are presented in table 4.

TABLE 4.—*Number of leafhoppers feeding on an artificially prepared food adjusted to 2 different pH values*

Experiment no.	Arrangement of food on membrane of cage	Length of feeding period	Average number of leafhoppers collecting over food at end of each 5-minute interval	
			Alkaline food (pH 8.5)	Acid food (pH 5.0)
		Minutes	*Number*	*Number*
1	In drops (36), in alternate rows of acid and alkaline food	50	7.9	2.0
2	Quadrants (membrane 8 cm in diameter), with acid food on opposite quadrants.	40	7.4	2.3
3	Quadrants (membrane 4 cm in diameter), with acid food on opposite quadrants.	40	7.0	2.9

It is evident that the leafhoppers concentrated on the food that was alkaline in reaction. They were observed, however, to explore the drops and quadrants of acid food by puncturing several times in different places and then to move on.

It was evident from observing the leafhoppers on the acid food, as recorded in table 4, that they were not feeding but were merely exploring the area.

Leafhoppers were also allowed to feed on single drops of solid media, one-half of which was acid and the other half alkaline. Such drops were formed as follows. To the melted media was added sufficient bromthymol blue to distinguish the acid from the alkaline medium. One large drop of the acid and one of the alkaline medium were deposited on a membrane, being separated only by a thin cover glass. The cover glass was removed when the media were partly solidified, and in this way direct contact was made between the acid and the alkaline food.

When leafhoppers were allowed to feed on such drops they explored the entire drop and then invariably fed from the alkaline side. The mouth parts could be seen in the alkaline food with the aid of a hand lens.

From the experiments presented thus far it is evident that the carbon dioxide treatment causes such striking chemical changes to take place in the beet plants that the leafhoppers are no longer able

to find the phloem. It is also evident that the leafhoppers prefer an alkaline food to one that is acid in reaction.

The implication is that under normal conditions the leafhoppers are guided to the phloem by a chemical gradient and that this gradient is so profoundly affected by the carbon dioxide treatment that in treated plants it is no longer present to direct the leafhoppers to the phloem; consequently, the insects are unable to find the phloem except by chance. As a result only an occasional infection is produced (table 2).

A consideration of the facts presented led to the hypothesis that this chemical gradient is in reality a pH gradient and that it is upset by treating the plant with a high concentration of carbon dioxide.

The evidence that led to these hypotheses is as follows: (1) The phloem contents of the beet petiole are alkaline while the cell sap of the parenchyma is acid in reaction; (2) the saliva of the leafhopper has been found to be alkaline in reaction (*5*) (which is significant, because the leafhopper must penetrate acid tissue in order to reach the alkaline phloem on which it feeds; in other words, the leafhopper's mouth parts are in an extremely sour medium as compared with its own saliva); (3) on treatment with high concentrations of carbon dioxide, the reaction of the parenchyma becomes more alkaline (*6*), while the phloem content becomes less alkaline or even acid, as will be shown later; (4) such a change in the reaction of the parenchyma and the phloem would upset any pH gradient that might exist; (5) the majority of the trails left by the leafhoppers while feeding on the normal petiole terminate in the phloem tissues; (6) the leafhoppers even changed the direction of their mouth parts in the parenchyma in order to reach the phloem in normal plants (table 3; Bennett (*1*) has observed this to be a very common occurrence); (7) only a very small percentage of the trails left by the leafhoppers while feeding from petioles treated with carbon dioxide terminated in the phloem tissues; (8) not one of the trails found in the treated petioles indicated that any leafhopper had changed the direction of its mouth parts in the parenchyma in order to feed from the phloem; (9) the leafhopper prefers an alkaline food to one that is acid in reaction (table 4).

THE pH GRADIENT IN NORMAL AND IN CARBON DIOXIDE-TREATED SUGAR-BEET PETIOLES

To test the hypotheses that the leafhoppers are guided to the phloem by a pH gradient and that this gradient is upset by carbon dioxide treatment, a microquinhydrone electrode was devised to measure the pH value of the cell sap of individual cells. Such measurements would reveal the nature of the pH gradient from the phloem to the epidermis of the petiole in the sugar-beet petiole.

A platinum needle sealed into a 3-mm glass tube was sharpened first by means of a file, then a hone, until a sharp, gradually tapering point was obtained. The sharpening was completed by placing the needle on a hone, where it was stroked (toward the point) with a rubber eraser containing powdered pumice. By this procedure a needle was made having a gradual taper to the point, which was 40μ in diameter.

The average diameter of the cells of the parenchyma of large beet petioles of the type used were found to be 150μ with only 10 percent

falling below 100μ. Consequently, the microelectrode could be inserted into a single cell without rupturing or disturbing the adjoining cells.

The microelectrode was clamped in one of the arms of a micromanipulator, making movement in any direction possible. A large freshly cut petiole, 3 inches in length, was mounted in a perpendicular position in the other arm of the micromanipulator. The lower end of the petiole was immersed in a saturated solution of potassium chloride, which served as a salt bridge and was in turn connected to a standard half cell. A dissecting microscope was mounted in front of the micromanipulator at an angle of approximately 25° from the perpendicular to allow the microelectrode to be mounted in a perpendicular position. The microscope was focused on the surface cells of the section of the petiole, and the tip of the microelectrode was placed

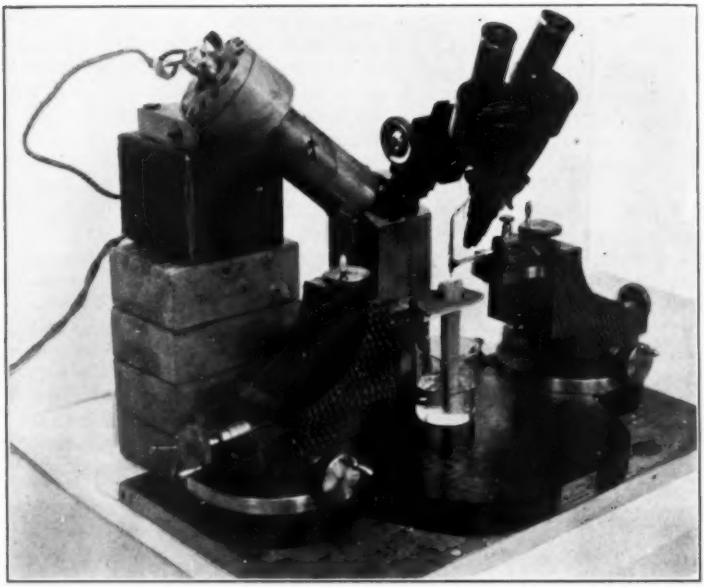

FIGURE 2.—Apparatus used to measure the pH value of the cell sap of individual parenchyma cells of sugar-beet petiole.

in the center of the field of view and near enough to the surface of the petiole to be in focus also. The apparatus described is shown in figure 2.

To determine the pH value of any cell or tissue of the petiole a fresh surface was cut, washed with distilled water, and dried with a filter paper. The microscope was focused on the surface cells of the petiole, which was then moved until the desired cells were in the microscope field. A minute amount of finely ground quinhydrone was sprinkled with a small camel-hair brush on the area to be tested. With the tip of the microelectrode in the center of the field and also in focus, the petiole was then moved until the desired cell was directly

below the microelectrode. The microelectrode was then lowered until it punctured the cell wall. The cell sap, being under slight pressure, forced its way past the microelectrode, thereby filling the cup formed by the cut cell above in which the quinhydrone was deposited. In order to get the quinhydrone into the punctured cell it was only necessary to withdraw the microelectrode. The particles of quinhydrone, being wet by the cell sap, could be seen to sink through the hole made by the microelectrode. The microelectrode was then lowered and the potential measured.

Because of the high resistance offered by the petiole it was necessary to employ a very sensitive galvanometer to measure the potential difference. The galvanometer used had a sensitivity of 0.00097 microampere per millimeter (scale at 1 m).

It required approximately 3 minutes from the time a fresh section was cut to determine the pH gradient, starting from the phloem and going to the epidermis of the petiole, or the reverse. The pH gradient was determined in different sections of the same petiole. Measurements were also made on a large number of petioles collected on different days.

The curve of figure 3 is typical of the measurements made of the pH gradient in the petioles of the normal sugar beet. The pH gradient was also measured in petioles that had been subjected to 75-percent carbon dioxide for 16 hours and in petioles that had been treated with the gas and allowed to recover (fig. 4).

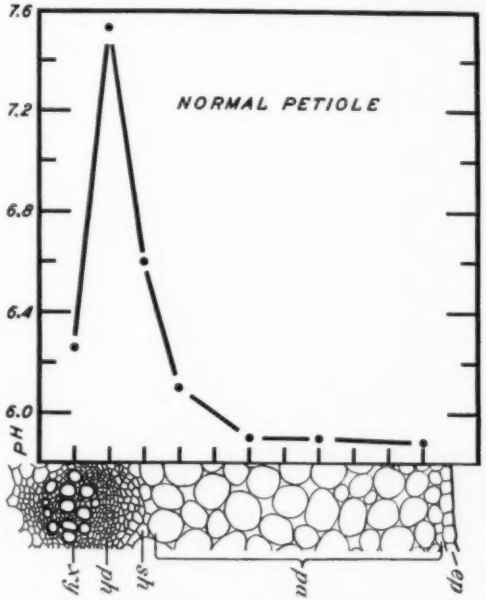

FIGURE 3.—The pH gradient in normal sugar-beet petiole: *xy*, Xylem; *ph*, phloem; *sh*, bundle sheath; *pa*, parenchyma; *ep*, epidermis

The pH value of the individual parenchyma cells was found to differ only slightly from that of the extracted juice as measured by the glass electrode. For example, by means of the glass electrode the mean pH value of the parenchyma beyond the fifth cell from the phloem tissue was found to be 5.9 and the pH value of the extracted juice of the same lot of petioles was found to be 5.7. A considerable number of pH measurements on the phloem exudate by colorimetric and potentiometric methods have been made. A pH value of approximately 8 was found in every case. Inasmuch as the electrode

crushes a small number of cells (among which are phloem parenchyma cells) when inserted into the phloem tissue, the pH values obtained are low as compared to the pH value of the contents of the sieve tubes. It is very probable that the pH value of the cell sap of the phloem parenchyma is considerably lower than that of the sieve-tube contents.

It is evident that a definite gradient exists out to the fourth or fifth parenchyma cell, the maximum change in pH value being about 2 units, or a hundredfold increase in the hydrogen-ion concentration, going from the contents of the phloem to the cell sap of the parenchyma cells near the epidermis.

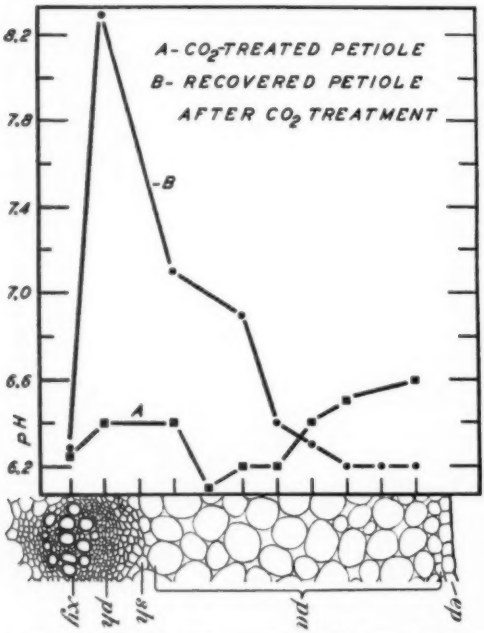

FIGURE 4.—*A*, pH gradient in a sugar-beet petiole treated with carbon dioxide; *B*, pH gradient in a recovered petiole, after carbon dioxide treatment. *xy*, Xylem; *ph*, phloem; *sh*, bundle sheath; *pa*, parenchyma; *ep*, epidermis.

The hydrogen-ion concentration of the phloem contents (fig. 4, *A*) has been increased approximately a hundredfold (from a pH of 8.2 to 6.2) by carbon dioxide treatment. The normal pH gradient has been entirely upset and even reversed.

The reversal of the gradient is probably due to the amount of carbon dioxide absorbed by the cells. The partial pressure of carbon dioxide would be greater in the cells immediately below the epidermis; consequently the reactions (*6*) producing hydroxyl ions would proceed further. The cells near the vascular tissue, far removed from the source of carbon dioxide, would alter their hydrogen-ion concentration but little. In this way the pH gradient may for a short period even be reversed.

If the pH gradient is the means by which the leafhoppers are guided to the phloem, then it is clear why such a large percentage of the trails in the petioles treated with carbon dioxide terminated in the parenchyma immediately below the epidermis. This is the point at which the hydrogen-ion concentration is least. If the leafhoppers penetrated deeper, the hydrogen-ion concentration would become greater instead of less, as in the normal plant.

It may be pointed out that in the cases where the leafhoppers changed the direction of their mouth parts in the parenchyma in order to feed from the phloem, they began changing direction approximately at the third or fourth cell from the bundle sheath. The

evidence strongly indicates that it is the pH gradient which guides the leafhoppers to the phloem in the normal plant.

DISCUSSION

In view of the alkaline reaction of the saliva of the leafhopper and the acid reaction of the parenchyma of the beet plant, it seems logical that a gradual pH gradient from the epidermis to the phloem would be instrumental in directing the leafhopper to the phloem. When the leafhoppper punctures the first cells below the epidermis, an enormous difference in hydrogen-ion concentration exists between the alkaline saliva of the leafhopper and the acid cell sap of the parenchyma. For example, if the pH value of the leafhopper's saliva is assumed to be 8.5 and that of the cell sap of the parenchyma to be 5.8, the cell sap taken up by the leafhopper would have a hydrogen-ion concentration 800 times greater than the saliva of the leafhopper. It seems probable that such a difference would have a pronounced physiological effect upon the leafhopper.

As the leafhopper's mouth parts penetrate deeper into the parenchyma, the difference in hydrogen-ion concentration decreases slowly. When the mouth parts approach to within three or four parenchyma cells of the bundle sheath (where the change in the pH gradient becomes very marked), the difference between the hydrogen-ion concentration of the cell sap and that of the saliva decreases rapidly. When the mouth parts reach a sieve tube, the difference between the hydrogen-ion concentration of the sieve-tube contents and that of the saliva becomes negligible, a condition preferred by the leafhopper.

Brandes (*3*), Davidson (*4*), Horsfall (*7*), and Smith (*13*) have shown that certain species of *Aphis* feed from the phloem tissues, but no explanation of the mechanism which directs the insect to these tissues has been offered.

It is quite evident from these investigations that it is no mere matter of chance that so large a proportion of the leafhoppers reach the phloem while feeding on the normal beet plant.

The idea of a pH gradient is not new. Sachs (*11*), in 1862, demonstrated that the phloem contents were alkaline to litmus.

Pearsall and Priestley (*8*) found the pH value of the xylem to be from 4.3 to 5.0 and, recalling that Sachs pointed out that the phloem may be as high as 7.8 or higher, remarked that the two cambial meristems of the normal dicotyledon lie across a marked pH gradient of hydrogen-ion concentration. The generalization was made that cambial activity takes place where there is a pH gradient, as from acid xylem to alkaline phloem. No measurements were made, however, to show the type and extent of the gradient that exists.

Priestley (*9*) discussed the general occurrence of phellogen, vascular cambium, and apical meristems and the origin of adventitious apical meristematic organizations in the light of the existence of such a pH gradient.

Rea and Small (*10*) and Small (*12*) made a very extensive general survey of tissue reactions by the range-indicator method. This method at best can only indicate roughly the general range in which the different tissues lie. With this method these investigators pointed out that the general pH gradient between phloem walls and xylem walls varies from 0.4 to 1.5 pH units in range.

Most of the modern theories attempting to explain growth and the metabolic processes of individual cells or tissues recognize the vital importance and influence that the hydrogen-ion activity may exert on the dynamic forces operating within the cell.

The unique mechanism the cells have for altering the hydrogen-ion activity of their own cell sap in the presence of high concentrations of carbon dioxide may serve admirably in studying certain of the fundamental factors involved in the metabolic processes within the cell and meristematic activity.

SUMMARY AND CONCLUSIONS

A study has been made of the relation of cell-sap reaction to curly top resistance in the sugar beet. As preliminary studies indicated a possible relation between hydrogen-ion concentration and the disease, sugar beets were treated with carbon dioxide.

Only a small percentage of the sugar-beet seedlings became infected with curly top when exposed to a high concentration of carbon dioxide preceding and during the inoculating period. The ratio of infection in the normal plants to that in the plants treated with carbon dioxide was found to be 4.7:1. Exposure of sugar-beet seedlings to high concentrations of carbon dioxide immediately after inoculation failed to reduce the percentage of infection below that of untreated controls.

When the curly top vector, *Eutettix tenellus* (Baker), feeds on the petioles of sugar-beet plants treated with carbon dioxide, it apparently loses its sense of direction, and its reaching the phloem, from which the insect normally feeds, is then a mere matter of chance. Fifty-six percent of the trails observed in the normal petioles terminated in the phloem, whereas only 12 percent of the trails found in the treated petioles terminated in this tissue. The ratio is 4.6:1.

The striking agreement between this ratio and the one noted in the second paragraph of this summary is further evidence that the virus must be deposited in the phloem in order to insure infection. The curly top vector, *Eutettix tenellus*, prefers an alkaline food (pH 8.5) to one that is acid in reaction (pH 5.0).

A microquinhydrone electrode was built in such a way that the pH value of the individual parenchyma cells of the sugar-beet petiole could be measured without disturbing adjoining cells.

A gradual pH gradient was found in the normal petioles of the sugar beet. By exposing beet petioles to high concentrations of carbon dioxide, the normal pH gradient was entirely upset and even reversed. The pH gradient in the petioles treated with carbon dioxide returned to normal when placed under atmospheric conditions.

The evidence indicates that leafhoppers feeding under normal conditions are probably guided to the phloem by the pH gradient.

Evidence of what appeared at first to be curly top resistance induced by pH changes in the host revealed that the apparent resistance of the beet plants could be attributed to the failure of the vector to inoculate plants that were under the influence of carbon dioxide. The carbon dioxide treatment upsets the normal pH gradient in the beet plant, which, evidence strongly indicates, is the means by which the leafhopper is able to locate the phloem.

LITERATURE CITED

(1) BENNETT, C. W.
1934. PLANT-TISSUE RELATIONS OF THE SUGAR-BEET CURLY-TOP VIRUS. Jour. Agr. Research 48: 665–701, illus.

(2) ———
1935. STUDIES ON PROPERTIES OF THE CURLY-TOP VIRUS. Jour. Agr. Research 50: 211–241, illus.

(3) BRANDES, E. W.
1923. MECHANICS OF INOCULATION WITH SUGAR-CANE MOSAIC BY INSECT VECTORS. Jour. Agr. Research 23: 279–284, illus.

(4) DAVIDSON, J.
1923. BIOLOGICAL STUDIES OF APHIS RUMICIS LINN. THE PENETRATION OF PLANT TISSUES AND THE SOURCE OF THE FOOD SUPPLY OF APHIDS. Ann. Appl. Biol. 10: 35–54, illus.

(5) FIFE, J. M.
1932. A METHOD OF ARTIFICIALLY FEEDING THE SUGAR-BEET LEAFHOPPER. Science (n. s.) 75: 465–466, illus.

(6) ——— and FRAMPTON, V. L.
1935. THE EFFECT OF CARBON DIOXIDE UPON THE pH AND CERTAIN NITROGEN FRACTIONS OF THE SUGAR-BEET PLANT. Jour. Biol. Chem. 109: 643–665, illus.

(7) HORSFALL, J. L.
1923. THE EFFECTS OF FEEDING PUNCTURES OF APHIDS ON CERTAIN PLANT TISSUES. Pa. Agr. Expt. Sta. Bull. 182, 22 pp., illus.

(8) PEARSALL, W. H., and PRIESTLEY, J. H.
1923. MERISTEMATIC TISSUES AND PROTEIN ISO-ELECTRIC POINTS. New Phytol. 22: 185–191.

(9) PRIESTLEY, J. H.
1928. THE MERISTEMATIC TISSUE OF THE PLANT. Biol. Rev. and Biol. Proc. Cambridge Phil. Soc. 3: 1–20, illus.

(10) REA, M. W., and SMALL, J.
1927. THE HYDRION CONCENTRATION OF PLANT TISSUES. Protoplasma 2: 45–58, illus.

(11) SACHS, J.
1862. UEBER SAURE, ALKALISCHE UND NEUTRALE REAKTION DER SÄFTE LEBENDER PFLANZENZELLEN. Bot. Ztg. 20: 257–265. [Original not seen.]

(12) SMALL, J.
1929. HYDROGEN-ION CONCENTRATION IN PLANT CELLS AND TISSUES. 421 pp., illus. Berlin.

(13) SMITH, K. M.
1926. A COMPARATIVE STUDY OF THE FEEDING METHODS OF CERTAIN HEMIPTERA AND OF THE RESULTING EFFECTS UPON THE PLANT TISSUE, WITH SPECIAL REFERENCE TO THE POTATO PLANT. Ann. Appl. Biol. 13: 109–139, illus.

FURTHER STUDIES ON THE RELATION OF THE CURLY TOP VIRUS TO PLANT TISSUES[1]

By C. W. BENNETT, *pathologist, Division of Sugar Plant Investigations, Bureau of Plant Industry, United States Department of Agriculture*, and KATHERINE ESAU, *instructor and junior botanist, University of California*

INTRODUCTION

As outlined in previous papers (*2*, *5*, *6*, *7*),[2] considerable evidence has been obtained that shows a very close relationship between the curly top virus and the phloem tissue of affected plants. This evidence is based on (1) anatomical studies that indicate restriction of necrosis to the phloem, (2) results of ringing experiments with *Nicotiana tabacum* L. and *N. glauca* (Graham) that indicate inability of the virus to pass an area devoid of phloem, and (3) determinations of relative amounts of virus picked up by the insect vector, *Eutettix tenellus* (Baker), when forced to feed on different tissues of the beet plant.

Improvement in methods for the recovery of virus from plant tissues has permitted a more extensive study of the virus content of different types of tissues and has provided a means of verifying the accuracy of previous determinations in which the virus content of tissues was measured by the amount of virus the insect vector was able to obtain by direct feeding. The results of some further studies on the relation of the curly top virus to different organs and tissues of beet, *Beta vulgaris* L., and tobacco, *Nicotiana tabacum*, are presented in the following report.

In these studies a special effort was made to obtain additional evidence bearing on the extent to which the curly top virus is restricted to the phloem tissue of affected plants.

MATERIAL AND METHODS

The beet plants used in these studies were of the curly top resistant variety U. S. 33. Seeds were planted in the fall of 1933 and 1934 and the tests were made on nearly mature plants in July, August, and September, of 1934 and 1935. Infection was from field sources but plants selected for the study were infected prior to the beginning of seedstalk formation, as indicated by the presence of symptoms on leaves below the base of the seedstalks. The symptoms on these resistant plants were relatively mild, and the disease did not markedly interfere with plant development. Numerous experiments have indicated that the concentration of recoverable virus in plants of this type is somewhat lower than that in plants injured more severely. However, the recovered virus was of a virulent type and produced severe symptoms on seedling beets of susceptible varieties.

The tobacco plants selected for study were of the Turkish variety. They were grown under greenhouse conditions and inoculated wit

[1] Received for publication Mar. 31, 1936; issued December 1936.
[2] Reference is made by number (italic) to Literature Cited, p. 620.

stock strains of the curly top virus. With only a few exceptions, organs and tissues that developed after the plants became infected were selected for tests.

The method employed in the tests of virus content of various organs and tissues of these plants was described in a previous publication (*3*). Juice was expressed from the tissues to be tested and added to an equal volume of 95-percent alcohol. The resulting precipitate was thrown down by centrifugation, washed once with 50-percent alcohol, dried, mixed with a volume of 5-percent sugar solution equal to the original volume of the juice, and centrifuged, and the supernatant liquid was used to feed nonviruliferous leafhoppers. After a feeding period of 4 to 6 hours, the leafhoppers were caged singly on beets for 7 days, and the number of plants infected by these leafhoppers was taken as a relative measure of the virus content of the tissue from which the original juice was derived. The preparations, regardless of the original source of the juice, provided a relatively uniform food material for the beet leafhopper. This method was used for all parts from which juice could be extracted. For certain structures, such as seeds and pollen, modifications of the method were introduced. These are described in later sections of this paper.

From both beet and tobacco, representative samples of the organs and tissues tested for virus content were selected for histological studies. These samples were killed in a chromo-acetic-formalin solution (*5*) and embedded in paraffin after dehydration in ethyl alcohol and clearing in butyl alcohol.

STUDIES ON SUGAR BEET

RELATION OF THE VIRUS TO TISSUES OF VEGETATIVE PARTS

In a consideration of the relation of the curly top virus to the plant, three types of tissues seem of major importance: (1) Meristem, (2) phloem, and (3) parenchyma. Studies on the distribution and concentration of virus in the plant are limited (1) to observations on occurrence and nature of pathologic changes indicating presence or absence of virus in different tissues and (2) to a recovery of the virus from these tissues by various means. As yet, not much evidence has been obtained on the relation of the curly top virus to meristem, but studies on parenchyma and phloem have been more successful.

The curly top virus occurs in the phloem in high concentrations. Evidence indicates a much lower concentration in parenchyma. Undoubtedly a large part of the virus that occurs in parenchyma is present in the exudate that escapes into the intercellular spaces of this tissue from the phloem. The extent to which the virus is able to invade parenchyma and the relation of the virus to the protoplasm of parenchymatous cells are of special interest in the case of curly top because of the apparently close relationship of the causal agent to the phloem tissue of affected plants.

Anatomical Observations

Following infection, the phloem develops striking abnormalities leading in most instances to necrosis. In susceptible varieties phloem necrosis is very pronounced (figs. 1, 2), but in the resistant plants used in this study this symptom was very mild (fig. 3, *B*) or was absent entirely.

Susceptible plants usually show severe stunting of the various organs, a phenomenon indicatinghy poplastic condition of tissues. Resistant plants, however, show relatively little stunting, and frequently in very resistant individuals no stunting is evident.

Whether or not the diseased plants show hypoplasia, the parenchyma of the pith of the crown and stalk and the ventral parenchyma of the petiole seem to develop no cytological abnormalities that could be taken as an indication of presence of virus in these regions. Examination of living cells and of material treated with different killing

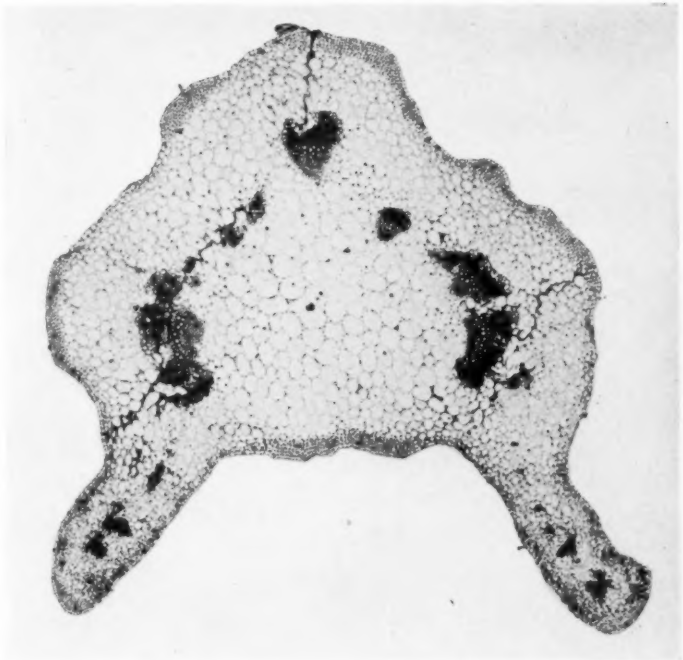

FIGURE 1.—Transverse section of a sugar-beet petiole from a plant severely affected by curly top. The phloem appears near the dorsal side of the petiole and shows severe necrosis accompanied by leakage of material to the surface of the petiole. × 23.

agents, including certain of those employed in mitochondrial technique, revealed no consistent differences between parenchyma cells of healthy and diseased plants.

In the leaves of severely affected susceptible plants, phloem necrosis is quite extensive and is frequently accompanied by exudation of material from the diseased phloem into intercellular spaces of the adjacent parenchyma and even to the exterior of the petiole and blades (fig. 1). When exudation is very abundant, the cells lying along the path of the material may separate from each other along the middle lamella (fig. 1, right), or may hypertrophy (fig. 1, above and left). Sometimes the exudate is not so abundant and fails to affect

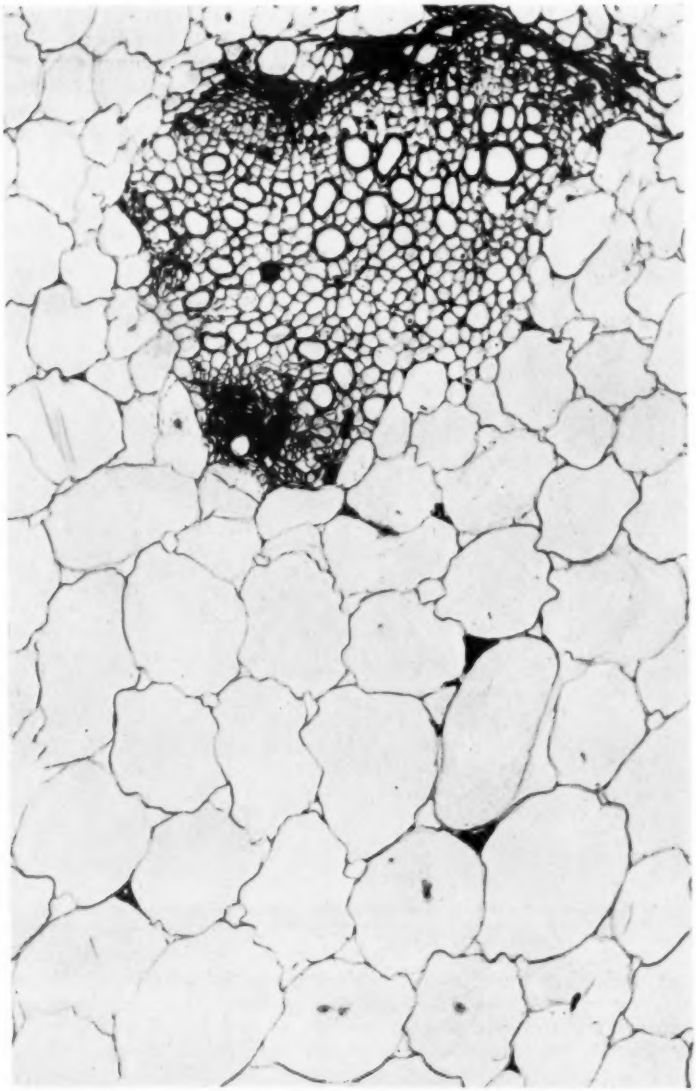

FIGURE 2.—Transverse section of a vascular bundle with adjacent ventral parenchyma from a diseased sugar-beet petiole. The bundle has phloem on both sides of the xylem. Black-stained exudate from the internal phloem group fills some of the intercellular spaces of parenchyma. × 150.

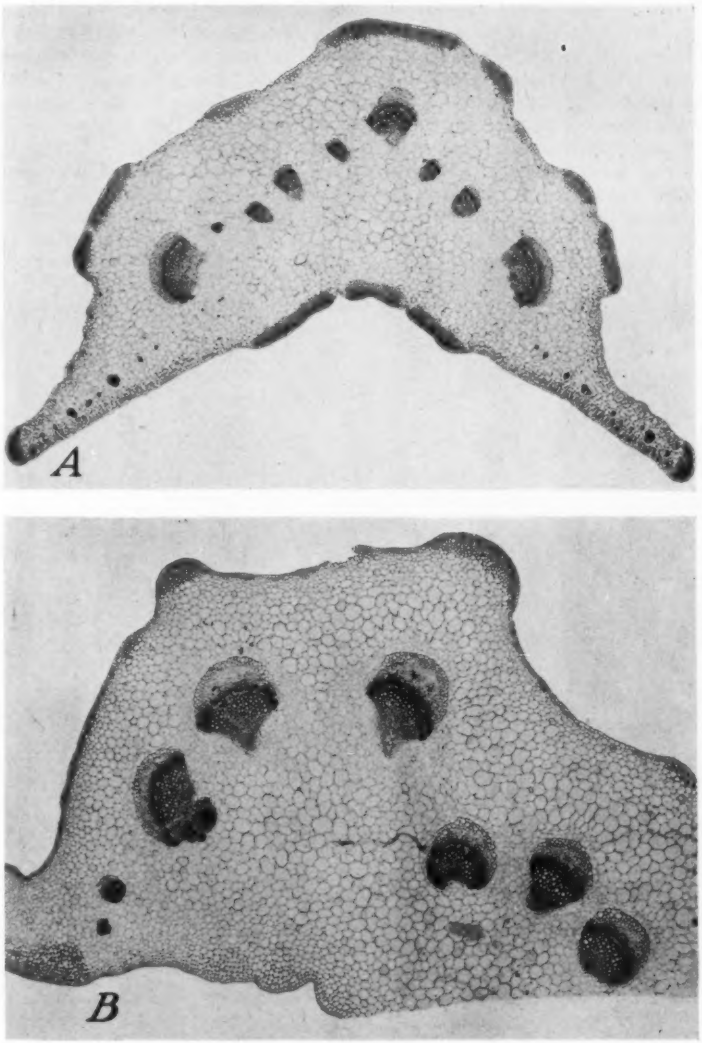

FIGURE 3.—*A*, Transverse section of a healthy sugar-beet petiole; *B*, transverse section of a portion of a petiole from a beet affected by curly top. Both petioles are placed with their dorsal sides above. Each vascular bundle shows xylem pointing toward the ventral side of the petiole, phloem and bundle cap toward the dorsal side. *B* shows some phloem necrosis. Both × 12.

the position and size of the cells between which it forces its way, but fills the intercellular spaces with darkly staining material (fig. 2). In the petiole most exudate moves from the phloem toward the periphery on the dorsal side, but some of it appears also in the intercellular spaces of the ventral parenchyma. This is particularly common near the extreme ends of the semicircle of bundles. Here the phloem swings around the xylem so that the bundle becomes bicollateral. Exudate from the internal phloem in figure 2 has moved into the intercellular spaces of the ventral parenchyma. The outer phloem, however, may also furnish some of the exudate that occurs on the ventral side. The presence of exudate in the ventral parenchyma of the petiole may be seen in figures 1, 2, and 4.

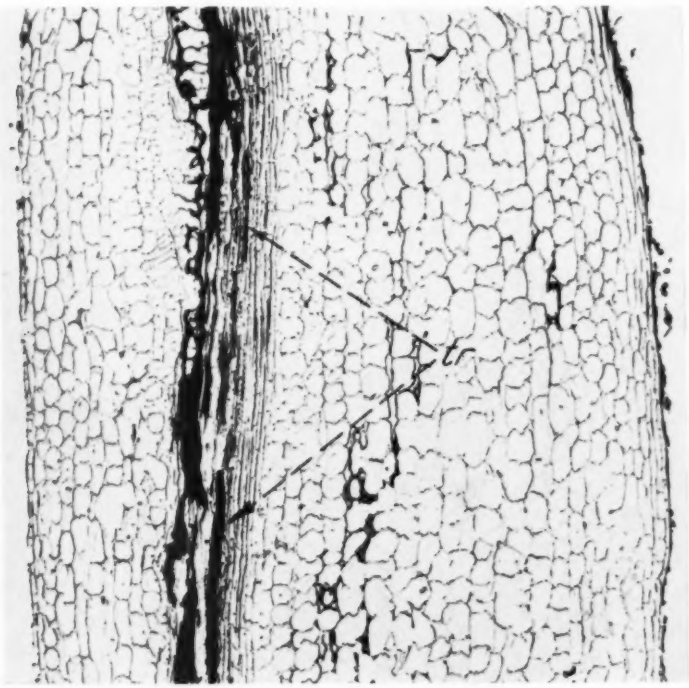

FIGURE 4.—Longitudinal section of a petiole from a diseased sugar beet. To the left is a vascular bundle with necrotic phloem. Within the xylem two sections of tracheae (*tr*) are filled with black-stained phloem exudate. The ventral parenchyma to the right of the bundle also contains such exudate in the intercellular spaces. × 46.

In resistant varieties of beets movement of material from the phloem into the intercellular spaces of the neighboring parenchyma is much less frequent than in susceptible varieties. When this occurs at all, the volume of material that moves into the parenchyma is relatively small and is confined, so far as observed, to areas in the leaf blade.

Infected plants of the resistant variety U. S. 33 were examined microscopically for the presence of phloem exudate in the intercellular

spaces of parenchyma of different parts of the plants. No exudate was found in the intercellular spaces of the parenchyma of the crown and flower stalk or in the ventral parenchyma of the petiole (fig. 3, *B*). It is not known that phloem exudate can be detected by microscopic examination in all cases, but, on the basis of the available evidence, it is considered that probably the parenchyma of selected individuals of this variety is as free from intercellular exudate as that of any other variety that might be selected.

Tests for Virus

The most favorable plant parts from which to obtain tissue free from phloem are the pith of the crown (fig. 5, *A*), parenchyma of the ventral side of large petioles (fig. 5, *B*), and pith of the flowering stalk.

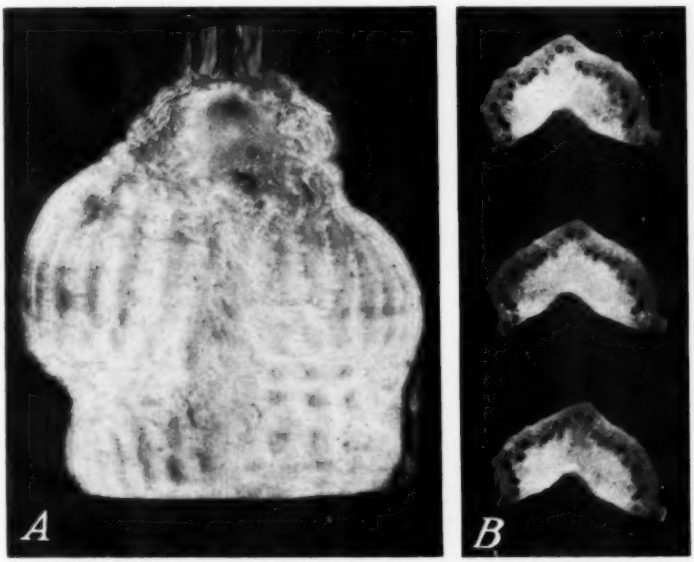

FIGURE 5.—*A*, Longitudinal section through the crown of a sugar beet showing the pith region above, which is free of vascular tissue; *B*, transverse sections through large sugar-beet petioles showing the vascular bundles near the periphery on the dorsal side of the petiole and a large parenchymatous area, free of vascular tissue, on the ventral side. × 1.

Vigorous plants with large petioles and fruitstalks were selected from field plots. The symptoms consisted mainly of vein swellings and indicated that the plants had a high degree of resistance. Blocks of parenchyma were removed from the crown, flowering stalk, and petioles of these plants. Some of these blocks were examined microscopically by means of freehand and prepared sections. No morphological or cytological abnormalities were observed and no phloem exudate was discovered in the intercellular spaces of the tissue of these regions.

Other blocks of parenchyma were removed and washed thoroughly in distilled water. The juice was expressed and its virus content was determined as already described. This virus content was compared

with that of equal volumes of juice from adjacent regions containing vascular tissue.

The results of these tests are shown in experiment 1 of table 1. They are in general similar to those obtained by feeding the leafhoppers directly on the tissues, and they support the concept that the curly top virus is much more concentrated in the phloem than in the parenchyma of the regions tested. No virus was obtained from the parenchyma of the petiole and in 20 of the 26 tests none was recovered from the parenchyma of the crown and flowering stalk. In the tests in which virus was recovered from parenchyma, its concentration appeared very much lower than in adjacent regions containing vascular tissue.

TABLE 1.—*Virus content of different organs and tissues of sugar-beet plants*

Experiment no.	Source of virus	Tests[1]	Tests that yielded virus	Plants inoculated	Plants infected	
		Number	*Number*	*Number*	*Number*	*Percent*
	Leaf	10	10	200	64	32.0
	Parenchyma of petiole	10	0	200	0	0
	Adjacent tissue	10	5	200	13	6.5
	Parenchyma of crown	12	1	240	5	2.0
1	Adjacent tissue	12	12	240	60	25.0
	Parenchyma of crown	6	2	120	3	2.5
	Adjacent tissue	6	6	120	56	46.6
	Parenchyma of stalk	8	3	160	3	1.8
	Adjacent tissue	8	8	160	47	29.3
2	Pericarp	16	14	320	59	18.4
	Seeds (mature)	16	16	320	205	64.0
3	Embryo	8	0	160	0	0
	Perisperm and seed coat	8	8	160	70	43.7
4	Seeds	4	4	80	37	46.2
	Seeds minus vascular region	4	4	80	17	21.2
	Vascular region of seeds	4	4	80	31	38.7
	Seeds	7	5	140	18	12.8
5	Upper half of seeds	7	1	140	1	.7
	Lower half of seeds	7	5	140	11	7.8
	Nonfilled seed coats	3	0	60	0	0
6	Embryo (germinated)	3	0	60	0	0
	Perisperm and seed coat	3	2	60	17	28.3
	Pericarp	6	5	120	18	15.0
7	Seeds (young)	6	1	120	1	.8
	Seeds (medium)	3	2	60	16	26.6
	Seeds (mature)	6	6	120	77	64.1

[1] 20 plants were inoculated in each test.

RELATION OF THE VIRUS TO FLOWER AND FRUIT PARTS

Symptoms of curly top are evident on the calyx, stamens, and pistils of flowers from infected plants. The external symptoms consist mainly of enlarged veins. Phloem degeneration occurs in all parts of the flower, hypertrophy and hyperplasia being evident as soon as sieve tubes are mature.

Unfortunately, the beet flower is too small to permit tests of its parts for relative virus concentration by the methods employed, so that little more than anatomical evidence is available regarding distribution of the virus in the flower parts. This evidence, however, indicates that the virus occurs in all the vascular bundles of the flower.

The beet seed is of considerable size and large enough to permit tests for virus content of its parts. Rather extensive tests were made to determine relative concentration and distribution of virus in the seed. In conjunction with these tests, anatomical studies were made to gain

an understanding of the normal anatomy of the mature seed and of the effect of the disease on the cytological and anatomical structure of the different seed parts.

Normal and Morbid Anatomy of the Seed

The sugar-beet seed is a flat lentil-shaped structure 1.5 to 2 mm in diameter. It lies horizontally in the ovarian cavity and the funiculus is bent under it. When a mature seed is removed from the green pericarp the funiculus is still intact and appears on the lower side of the seed as a small sharply bent stalk (fig. 6, *A*, *B*).

The embryo is curved around the perisperm, a starchy storage tissue of nucellar origin. In the mature seed only a small amount of endosperm occurs near the radicle (figs. 6, *C*; 7).

The embryo is rich in albuminous and oily storage materials. According to De Vries (*9*), starch is absent from the resting embryo but appears there during germination, when the embryo absorbs starch from the perisperm.

The seed coat has two layers of thick-walled cells enclosing between them some thin-walled parenchyma (figs. 7, 8). In the regions where the seed coat is thinnest only one or two layers of parenchyma cells occur, but near the chalaza they are more numerous. The vascular tissue of the seed is distributed within the parenchyma of the chalazal region.

The vascular tissue is composed of primary xylem and phloem. The strand that connects the ovule with the mother plant passes through the funiculus and enters the parenchyma lying between the inner and outer layers of the seed coat. Approximately midway between the outermost limit of the outer integument and the inner limit of the inner integument, the strand splits in two and forms a saddle-shaped structure which embraces the base of the perisperm (figs. 6, *A*, *B*, *D*; 8). The region traversed by the two branches of the funicular strand is referred to as the chalaza in this paper.

Within the funiculus proper the vascular strand is collateral (fig. 6, *E*), but within the chalaza and slightly below it the phloem occurs on all sides of the xylem (fig. 6, *F–H*). The phloem extends farther than the xylem, so that at the summits of the branches only phloem elements appear in section (fig. 6, *I*).

There is a heavy cuticle on the inner wall of the innermost layer of the seed coat. This cuticle may be identified by the use of Sudan III, when the embryo consists of only about nine cells. The cuticle stops abruptly in the chalazal region where the phloem approaches the perisperm (figs. 7, 8). In this manner a "passage region" is left between the chalaza and the perisperm through which materials may move from the phloem into the perisperm. The phloem, however, does not come in direct contact with the perisperm cells. A tightly packed layer of cells, remarkably rich in tannin compounds, intervenes between the phloem and the perisperm (figs. 6, *C–I*; 7; 8). The corners of these cells have prominent thickenings which show the characteristic fatty staining reaction with Sudan III. The deposition of this fatty substance occurs at the time the cuticle is laid down on the inner wall of the inner integument. When the seed is mature and the funiculus is dried up, the entire walls of the passage region show positive reaction with Sudan III. The embryo and perisperm appear to become sealed off in a suberized layer.

As in all other seeds, vascular connection does not exist between the embryo and the mother plant. But the embryo itself develops mature protoxylem elements in the cotyledons before the seed

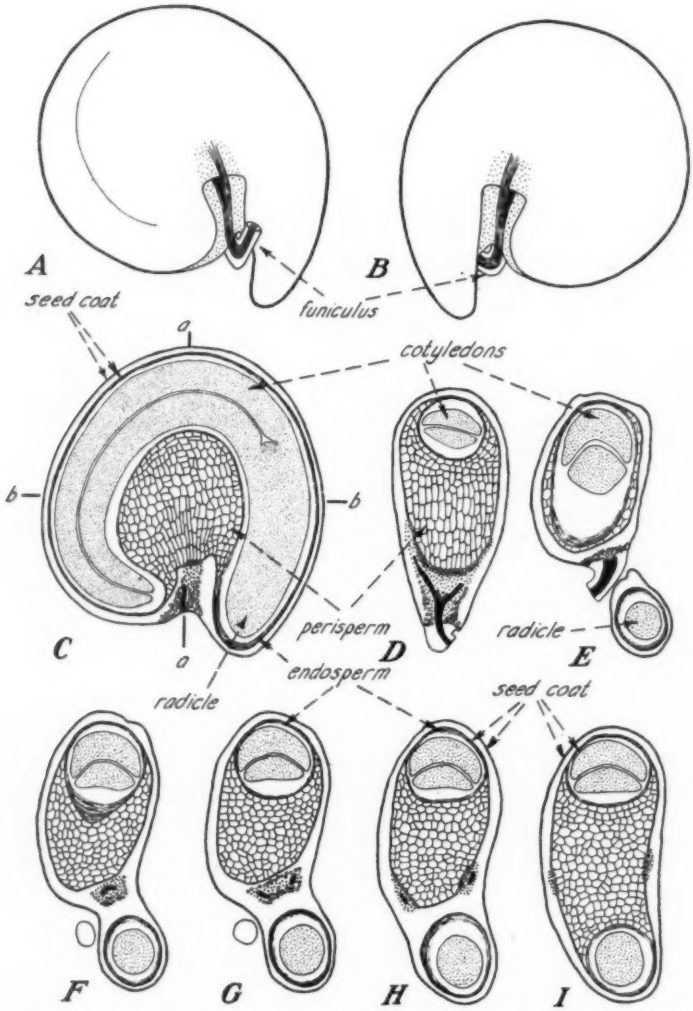

FIGURE 6.—Sugar-beet seed: *A* and *B*, Diagrams showing the distribution of the vascular tissues; *C*, section of mature seed, longitudinal with respect to the embryo; *D*, transverse section made along the axis *a* in *C*; *E–I*, successive transverse sections made parallel to the axis *b* in *C*, *E* being the lowest section. In *C–I*, the xylem is shown in solid black, the phloem by heavy stipples, and tannin-containing cells by circles. All × 18.

germinates. As to the phloem, in the material studied no fully matured sieve tubes were observed in the resting seed, but the young protophloem elements could be readily recognized by the presence of

vacuolating nuclei, sieve-tube plastids, and a coarse network of strands filling the lumina of cells. Sieve-plate perforations were not perceptible.

In partly germinated seed, in which the seed coats were still clinging to the embryo and the perisperm was not yet absorbed, mature sieve tubes were present in the embryo. They were free from nuclei and were "clear" because of the disappearance of the coarse network of strands, and they showed end-wall perforations.

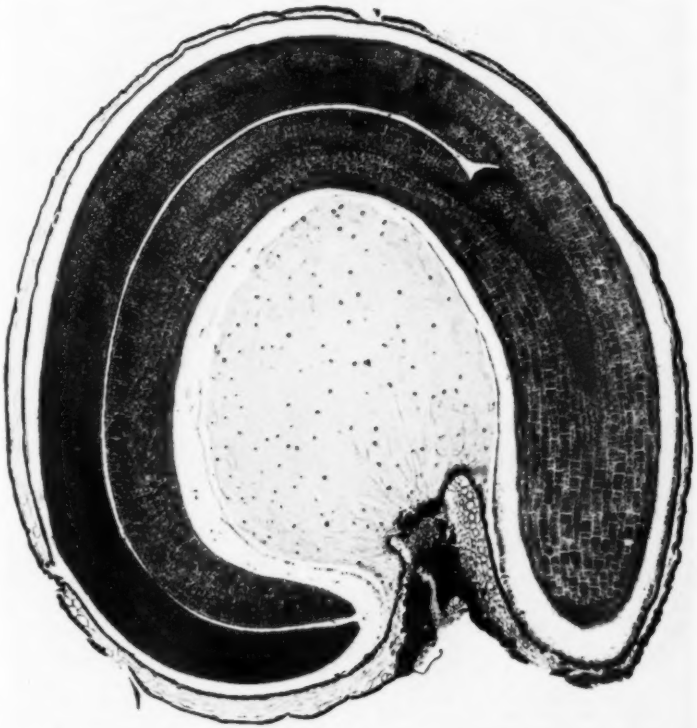

FIGURE 7.—Section through a healthy mature sugar-beet seed, longitudinal with respect to the embryo. The cotyledons are to the left, the hypocotyl to the right. The curved embryo partly encloses the perisperm. The chalaza is below. Compare also with figure 6, *C*. × 46.

Previous studies (*6*, *7*) indicate that curly top virus moves in mature sieve tubes. As the embryo develops such elements before it completes the absorption of perisperm, the virus probably could exist in the embryo if it managed to enter it with the absorbed food.

Studies of virus behavior have given strong evidence that continuity of cytoplasm is necessary to permit movement of virus from cell to cell. No obvious cytoplasmic connection, however, appears to exist between the embryo and the other parts of the seed, unless possibly through the suspensor. In young seeds the latter structure

was readily recognized, but in mature embryos degenerating or empty cells were found in place of the suspensor.

The mature embryo appears to lie free within the embryo sac and its prolongation, the caecum (*1*), as is shown in figure 7. The section in this figure, however, was not cut through the suspensor. It is impossible to obtain a perfect median section of a beet embryo because it is so curved that the tips of the cotyledons and that of the radicle lie in different longitudinal planes.

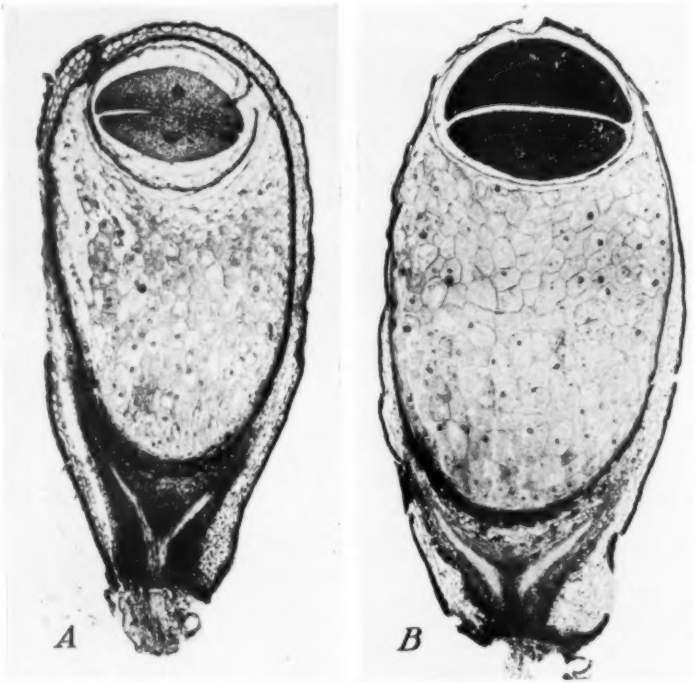

FIGURE 8.—*A*, Section through a healthy sugar-beet seed, transverse with respect to the embryo. Below is a portion of the funiculus, which merges into the chalaza with its forking xylem strand. The perisperm is in the middle, the cotyledons above. × 40. *B*, Section through diseased sugar-beet seed, slightly more mature than the healthy seed in *A*. The phloem appearing above the xylem fork shows hypertrophy. × 40.

One side of the embryo is adjacent to the perisperm; the other side is next to the inner layer of the seed coat. A thin lining of crushed nucellar cells separates the embryo from the perisperm and the inner layer of the seed coat except in the region of the radicle where some endosperm cells occur (fig. 7).

As already mentioned, phloem degeneration occurs in all parts of flowers from affected plants. In the ovule this degeneration becomes evident as soon as phloem develops. As in root tips or young leaves, the development of the first sieve-tube elements in the young floral parts is followed by degeneration of the phloem. Primary hyperplasia, which results in an increased production of sieve tubes, is

very conspicuous in the chalaza so that the phloem in that region is more massive in diseased than in healthy seeds. Degenerating phloem cells occur next to the "passage cells" intervening between the phloem and the perisperm, but the passage cells do not seem to be affected (fig. 8, *B*). No differences were recognized in the structure of embryos and perisperm from healthy and from diseased plants.

In many instances the perisperm cells of healthy and diseased seeds showed shrunken nuclei near the passage region (figs. 7 and 8, *A*) and the cytoplasm formed darkly stained coarse threads on which spherical bodies were strung. Although this fixation image is particularly common in diseased seeds, its occurrence in the healthy seeds seems to indicate that it is not a symptom of curly top.

Tests for Virus

Anatomical observations suggest that the virus invades the vascular area of the seed quite early in its development. To supplement these observations, attempts were made to recover virus from seeds and to determine its location and relative concentration in the different parts. The first tests were made to determine the virus content of the seed as a whole as compared with that of the surrounding pericarp. In these tests mature seeds from green plants were removed from the pericarp, ground in a mortar, mixed with distilled water, and centrifuged. The supernatant liquid was added to an equal volume of 95-percent alcohol, and the resulting precipitate was thrown down by centrifugation. The precipitate was dried, mixed with 1 cc of 5-percent sugar solution, and centrifuged. The supernatant liquid was tested for virus content in the usual way by means of leafhoppers. Seventy-five seeds were used in each test. The pericarps from which the seeds were removed were tested by the same method.

The results of these tests are shown in experiment 2 of table 1. Preparations derived from the pericarp gave considerable infection, but those obtained from the seeds gave a much higher percentage of infection. Mature seeds apparently have a very high concentration of virus.

Location of Virus in the Seed

As illustrated in figure 7, the beet seed is composed largely of perisperm and embryo. By splitting the seed coat at the top of the seed and applying pressure, the embryo may be removed without any apparent injury to it. Soaking the seed in water for a few minutes prior to splitting the seed coat facilitates this operation.

Embryos were removed from seeds from curly top plants and washed several times in distilled water. They were then ground in a mortar, mixed with distilled water, and centrifuged. The supernatant liquid was precipitated with alcohol, and the precipitate was tested for presence of virus as already described. The parts of the seed remaining after the embryo was removed were treated in a similar way, except that in some of these tests 0.5 cc of juice from a healthy beet was added to hasten precipitation with alcohol. The addition of this amount of beet juice apparently had no influence on the amount of virus that was recovered. Seventy-five seeds were used in each test.

The results of these tests are shown in experiment 3 of table 1. Embryos yielded no virus in any one of the eight repetitions of this experiment; whereas preparations from the parts of the seed remaining after the embryo was removed produced a high percentage of infection in seedling beets, showing that the virus in beet seeds is restricted to parts other than the embryo.

After it had been demonstrated that the embryo contains no virus, further tests were carried out to determine whether the virus of the seed occurs in the perisperm or is confined to the small amount of phloem at the basal end of the seed between the seed coat and the perisperm.

In each test 50 mature seeds were used. These were divided into 2 lots of 25 seeds each. One lot was not dissected and served as a control. With the other lot of 25 seeds, an attempt was made to separate the part of the seed containing the vascular elements from a part free from conductive tissue. Two methods were employed to effect this separation. In the first, the chalazal end and two sides of each seed were removed by means of a sharp razor. The parts removed consisted of part of the seed coat, the vascular system, and some of the embryo and perisperm. The remaining part consisted mainly of perisperm and embryo but contained a part of the seed coat. In the second method the seeds were split into two approximately equal upper and lower parts. The lower part contained the vascular elements but the upper one presumably was free from vascular tissue, since the vascular strands extend less than half the length of the seed. In all of these tests the seeds or seed parts were ground in a mortar, mixed with water, and centrifuged, and the supernatant liquid was treated as already described.

The results of these tests are shown in experiments 4 and 5 of table 1. In experiment 4, with seeds dissected by cutting away the parts containing vascular elements, a high concentration of virus is indicated for the seeds as a whole and for the parts containing the vascular tissue. However, considerable virus was obtained also from the parts presumably containing no vascular elements. In experiment 5, where the seeds were cut into two halves, infection was much too low to provide conclusive results.

These latter tests were made in August, during a period of very high temperatures, which probably accounts partly for the low percentage of infection. The results of these two experiments indicate that the place of greatest virus concentration in the seed is the basal end in the region containing the vascular system. They also suggest the probable occurrence of a certain amount of virus in the perisperm, although the possibility of including some vascular tissue with the perisperm in these tests was not eliminated entirely.

Full-size seed coats, apparently fully developed but with no perisperm or embryo, are found frequently in seed balls. A number of such seed coats were tested for virus in the usual way, 25 seed coats being used in each test. As shown in experiment 5 of table 1, no virus was recovered from this material.

Tests were made next to obtain evidence as to whether any virus passes into the embryo or the developing seedling during the process of germination. Mature seeds from infected plants were removed from green seed balls and placed on blotting paper in a moist chamber. The seeds germinated in from 2 to 5 days. When the larger seedlings

had attained a size in which the cotyledons had turned green, the seedlings were tested for virus content separately from the remains of the seed coats and perisperm. Under the conditions of these tests not all of the starch of the perisperm had been utilized by the seedlings. Seeds that did not germinate were discarded.

The results of these tests are given in experiment 6 of table 1. They show conclusively that seeds capable of germinating may contain large quantities of virus and that this virus is unable to enter the embryo or young seedling during the process of germination. There is no evidence of inactivation of virus in the process of germination of the seed. Some of the stored food was utilized and the virus was left behind in the seed coat and attached parts, where it remained in active condition for at least several days.

RELATION OF STAGE OF MATURITY OF SEEDS TO VIRUS CONTENT

The results of previous tests (*2*), in which nonviruliferous leafhoppers were allowed to feed on very young seeds from diseased plants, indicate that the virus is not present in young seeds in quantities sufficient to be detected by the direct feeding method. Since the mature seed has a very high virus content, it seems probable that there is a rapid increase in virus content as the seed matures.

An experiment was made to determine the accuracy of previous determinations of virus content of young seeds and to gain information as to the virus content of seeds in different stages of maturity. Seeds of three ages were used in these tests: (1) Seeds quite immature, seed coats not colored, fertilized eggs in first stages of division, no starch in the perisperm; (2) seeds with light-brown seed coats, embryos about one-third of their ultimate length, endosperm present, perisperm containing some starch; (3) mature seeds, seed coat brown, embryos full length, perisperm well filled with starch. Some of the mature seeds had withered funiculi. The seeds were removed from the pericarp and tested for virus content according to the usual procedure. Seventy-five seeds were used in each determination. The pericarps from which the seeds were removed also were tested.

The results of these tests (table 1, experiment 7) confirm those obtained in other experiments with young seeds and indicate a very low virus content in the seed in the early stages of its development. Evidently, as the seed matures its virus content increases rapidly and reaches a level that apparently is above that of any other part of the plant.

LONGEVITY OF THE VIRUS IN DRY SEEDS

Seeds from three plants affected by curly top were dried at room temperature and tested at intervals of 1 month to determine the length of time the virus remained in active condition. The dry seed balls were soaked in water for several hours before being tested for virus content. Then the seeds were removed from the pericarps, macerated in a mortar, and tested according to the procedure already described. Seventy-five seeds were used in each test.

The results of this experiment, which are given in table 2, show a considerable decrease in virus content in 1 month, a further decrease in 2 months, and apparent absence of active virus in seeds that were stored 3 and 4 months.

TABLE 2.—*Longevity of the curly top virus in dry beet seeds* [1]

Experiment	Plants infected by inoculum prepared from seeds after indicated number of months in storage				
	[2]0	1	2	3	4
	Number	*Number*	*Number*	*Number*	*Number*
1	15	7	0	0	0
2	13	8	5	0	0
3	16	4	5	0	0

[1] 20 plants were inoculated in each test.
[2] Tests were made before the seeds were dried, as a check.

One of the seedstalks from which seeds were removed was tested for virus content at monthly intervals for 7 months. The results of these tests indicate that the original virus content of the stalk was far below that of the seeds and that active virus content decreased with aging. However, virus was recovered in each of the seven tests, suggesting that virus may be inactivated less rapidly in tissues of the dry seedstalk than in dry seeds.

TESTS FOR SEED TRANSMISSION

Evidence that under ordinary field and greenhouse conditions curly top is not seed-transmitted is rather extensive and quite conclusive. Severin (*8*) grew 22,738 plants from seeds derived from infected plants and found no case of seed transmission. In the thousands of seedlings grown at the Riverside, Calif., station of the Division of Sugar Plant Investigations, occurrence of disease could in no case be traced to seed transmission, though many of the seeds used undoubtedly came from infected plants. Results of field observations furnish similar evidence.[3]

The observations in the field and in the greenhouse were made, however, on seedlings from seeds that were kept in storage for a time. It has been shown that the curly top virus loses its activity in dried seeds in less than 3 months. Obviously, seeds kept in storage longer than the period during which the virus remains active in stored seeds could produce only healthy seedlings, regardless of their virus content at an earlier date. To eliminate the possibility of inactivation by drying, seeds almost mature but having green pericarps were taken from infected plants and immediately tested for transmission of the virus through the seeds. In some cases entire seed balls were planted in sand. In others, the seeds were removed from the balls and germinated in sand or on blotting paper in moist chambers. The seeds required no rest period and germinated in from 2 to 5 days.

As shown in table 3, germination was relatively high and only healthy seedlings were produced by these seeds. Some of the seeds that failed to germinate on blotting paper were tested for virus content and proved to carry a high concentration of virus. The seed coats and the parts remaining in the seed coat after germination were tested for virus after the seedlings were about 1 inch long, and

[3] Supplemental evidence as to absence of seed transmission is afforded from experiments conducted in Michigan by G. H. Coons and J. E. Kotila, in which sugar-beet seed produced in New Mexico subject to severe exposure to curly top was used in seed-treatment experiments. More than 200,000 plants were concerned in these tests and no case of curly top was found, the plots being critically examined for any such occurrence. (Verbal communication.)

appeared to contain about as much virus as the seeds that did not germinate. These results show that the presence of large quantities of virus in seeds does not necessarily prevent germination, and suggest that the virus content of seeds has no influence on germination. Also, they furnish evidence that the embryo developing into a seedling is able to drain the seed of stored food without acquiring any of the virus that is either stored with the food or present in the immediate vicinity of the stored food.

TABLE 3.—*Results of tests for seed transmission of the curly top virus*

Treatment of seed balls or seeds	Seed balls or seeds planted	Plants produced	
		Healthy	Diseased
	Number	*Number*	*Number*
Seed balls in soil	3,200	3,970	0
Seeds in sand	225	141	0
Seeds in moist chamber	150	134	0

STUDIES ON TOBACCO

The symptoms produced on tobacco by curly top are rather characteristic and marked. The first symptoms following infection are very severe. The young leaves show vein clearing, become much distorted, and remain small. The stem also is usually distorted in the region of attachment of first affected leaves. Soon the growing point of the infected plant recovers to a marked degree and frequently shows only translucent veins in the very young leaves in subsequent growth. Such partial recoveries are due to plant responses and not to attenuation of the virus. Although vein swelling is less marked in tobacco than in beet, usually it is perceptible on the calyx and fruits of affected plants. Surface exudate from the phloem, which is common in the beet, has not been observed in tobacco.

The tobacco plant was selected for a study of the relation of the curly top virus to different tissues because some information on the movement of virus was already available for this plant from previous ringing experiments (*2*). These indicate that the virus will not pass through the woody cylinder. Moreover, this plant is different from the beet in anatomical structure. The presence of internal phloem and absence of anomalous growth affords an opportunity for making determinations of virus content of two distinct phloem-containing regions as well as of woody tissue and pith. Also, its flowers are large enough for determination of virus content of the various parts. This is especially important in the determination of virus content of different regions of the anthers. Stamens of beet are too small for satisfactory use in such a study.

Since the liquid content of the phloem does not move to the surface of leaves and petioles in diseased tobacco plants, it seems probable that movement of phloem content into the intercellular spaces of the cells of tissues adjacent to the vascular bundles may not occur or may be less extensive than in beet. If no phloem exudate occurs in the intercellular spaces of parenchyma of tobacco, the amount of virus recovered from the juice of such tissue should indicate to some extent the virus content of the parenchyma cells themselves.

VIRUS CONTENT OF VEGETATIVE PARTS

Phloem degeneration in Turkish tobacco plants affected with curly top passes through stages similar to those observed in the sugar beet affected by the same disease (*6*), but the necrotic tissue shows less pronounced accumulations of darkly staining material (cf. figs. 1; 9, *B*). In the final stages of degeneration the collapse of cells leads to a formation of internal cavities that may be seen in freehand sections as well as in paraffin preparations. Figure 9, *A*, shows a leaf vein from healthy tobacco and figure 9, *B*, shows a vein from a comparable leaf affected by curly top. The collapse of cells in the diseased leaf is perceptible in both the external and the internal phloem. The xylem is slightly hypoplastic.

Leaves and different parts of the stem were tested for their virus content. Plants used for these studies had been inoculated by the grafting method and were allowed to grow until they were almost mature. Except in two tests, leaves and stems selected for the test were produced after the plants were infected. Entire leaves were used, and the stems were dissected into (1) bark,[4] (2) wood, (3) internal phloem including considerable pith, and (4) pith. Tests were made from preparations from expressed juice as already described.

The results of these tests are shown in experiment 1 of table 4. They indicate that juice derived from the bark and from the internal phloem had a relatively high virus content, whereas that derived from the wood and pith had a rather low virus content. These results are similar to those obtained from the vegetative parts of beets (table 1, experiment 1), although the percentage of infection from wood and pith of tobacco was somewhat higher than that from parenchymatous tissue of beet.

TABLE 4.—*Virus content of different organs and tissues of tobacco plants*

Experiment no.	Source of virus	Tests [1]	Tests that yielded virus	Plants inoculated	Plants infected	
		Number	*Number*	*Number*	*Number*	*Percent*
1	Leaf	6	6	120	35	29.1
	Bark	9	8	180	41	22.7
	Wood	9	3	180	7	3.8
	Internal phloem	9	6	180	56	31.1
	Pith	9	3	180	8	4.4
2	Calyx	3	2	60	26	43.3
	Corolla	3	2	60	18	30.0
	Filaments	3	3	60	13	21.6
	Anthers	2	2	40	12	30.0
	Pistils	4	1	80	1	1.3
3	Pollen	5	0	100	0	0
	Anther (remains)	2	2	40	12	30.0
	Parenchyma of anthers	9	0	180	0	0
4	Phloem of anthers	9	3	180	5	2.7
	Anthers	4	2	80	4	5.0
	Capsule wall	2	2	40	4	10.0
5	Placenta	3	3	60	22	36.6
	Seeds	6	4	120	8	6.6

[1] 20 plants were inoculated in each test.

[4] The term "bark" as used in this paper signifies all tissues of the stem from the epidermis to the cambium, inclusive.

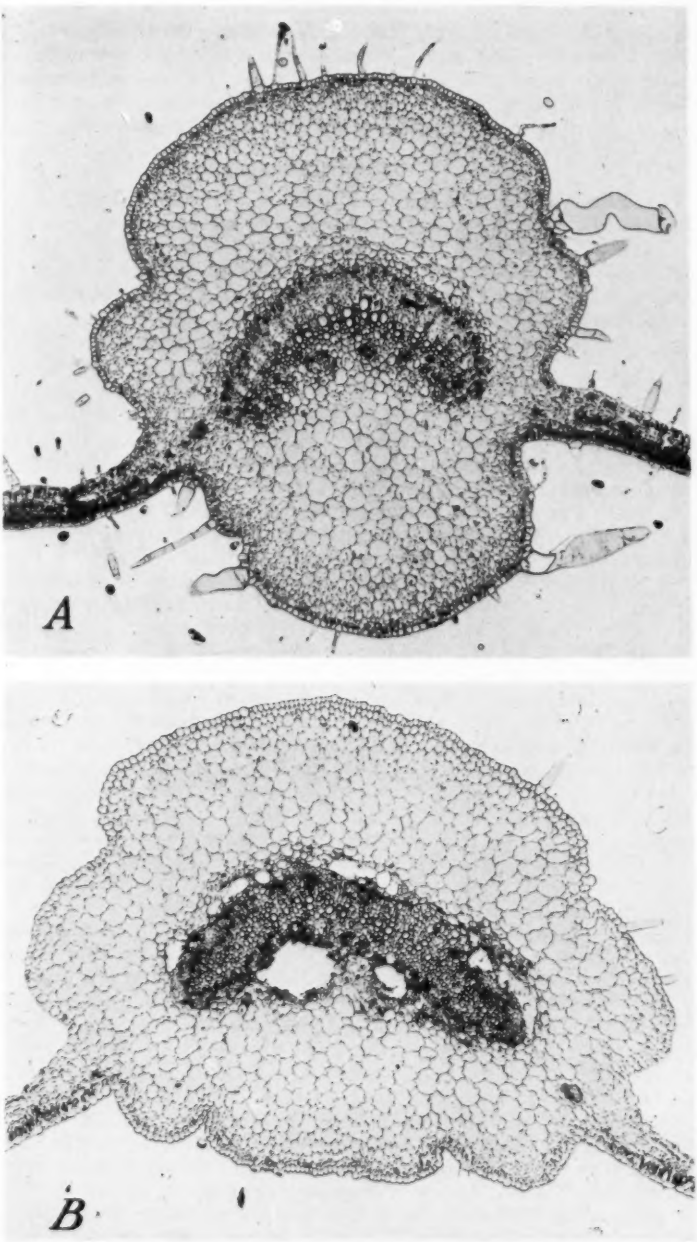

FIGURE 9.—Transverse sections of midribs of leaves of *Nicotiana tabacum: A*, From a healthy leaf; *B*, from a diseased leaf. The phloem in *B* is in advanced stage of degeneration; it shows collapse of cells but no phloem exudate. × 41.

VIRUS CONTENT OF FLOWER AND FRUIT PARTS

The calyx, corolla, pistil, and stamens of the tobacco flower show distinct vein swelling and a certain amount of distortion, even in plants that otherwise appear healthy. Phloem degeneration is evident in all flower parts of diseased plants, and occurs even in the smallest bundles in which only a few sieve tubes are present.

Virus Content of Flower Parts

The relative virus content of various parts of the flower was determined in flowers from plants that had been diseased more than 2 months. The flowers were dissected into calyx, corolla, anthers, filaments, and pistils. The tests of these organs were made in the usual way on preparations from expressed juice. The results given in experiment 2 of table 4 show that virus occurs in all of these structures, although relatively little was recovered from pistils.

FURTHER TESTS OF ANTHERS

Additional tests were made to determine in what part of the anther the virus is located, and, particularly, whether it occurs in the pollen. The tobacco anther contains considerable pollen and also furnishes rather favorable material from which to obtain tissue free of phloem elements. The vascular strand passing through the filament into the connective does not ramify but continues as a single strand almost to the apex. It is rather narrow in the filament, but widens out in the lower and middle parts of the connective, again becoming very narrow toward the apex of the anther. It is amphicribral in structure, the narrow xylem strand being surrounded by phloem.

Figure 10, *A* to *G*, represents transverse sections through the mature anther; whereas, figure 10, *H*, shows a whole anther seen from the dorsal side. The anther is divided longitudinally into a right and left lobe, each with two locules. At maturity, the connective tissue, which joins the two lobes and forms a partition between the two locules, separates from the side walls of the anther, as shown in *B* to *F* of figure 10. Each of the side walls bears a longitudinal line of dehiscence, indicated in the figure by very dense stippling.

Although the vascular and the sporogenous tissue of the anther are not in contact with each other, there are some thin-walled parenchyma cells that appear to serve as a connection between the two. These cells are represented by rather heavy stippling in *A* to *G* of figure 10. They make up the partition between the pollen sacs and form a narrow layer between this partition and the vascular strand. Similar thin-walled cells occur around the vascular strand also, but in figure 10 these cells are included with the vascular tissue in the cross-hatched circle. In young anthers these parenchyma cells are like the other sterile cells making up the anther walls, but in mature anthers they differ strikingly from the adjacent cells; they remain thin-walled, while the other cells of the anther walls develop the characteristic secondary thickening. The appearance and distribution of the thin-walled cells suggest that these cells are the tissue that conveys the materials from the phloem to the developing pollen.

Tests were carried out to determine whether virus passes out of the phloem of the connective into the surrounding tissue of the anther, especially into the pollen. About 0.1 g of pollen, which was

shaken from mature anthers, was used in each test. The anthers from which the pollen was taken were also tested for virus. The material was ground in a mortar, mixed with water, and centrifuged, and the supernatant liquid was tested as in the case of the expressed juice.

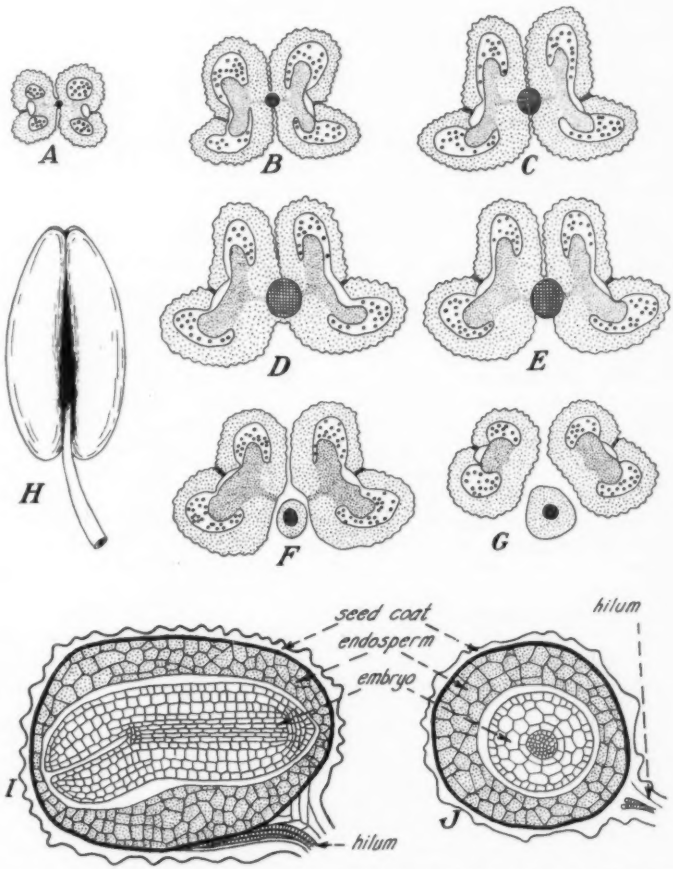

FIGURE 10.—*A–G*, Transverse sections made at different levels through an anther of healthy *Nicotiana tabacum*, (*A*) showing the upper, (*G*) the lower part of the anther. The cross-hatched circles indicate the position of the vascular tissue. × 22. *H*, General view of a tobacco anther seen from the dorsal side where the filament is attached. × 11. *I*, Longitudinal, and *J*, transverse section through a mature seed from a healthy plant of *Nicotiana tabacum*. × 71.

The results of these tests are given in experiment 3 of table 4. They show that virus was not recovered from the pollen, but was obtained from the anthers from which the pollen was removed.

Further efforts were made to recover virus from parts of the anther containing no phloem. Anthers not yet open were split longitudinally through the two halves, thus separating the outer tissue containing no vascular tissue from the part containing the

connective. In each test, 100 anthers were separated in this manner and the two parts were tested for virus as in the previous experiment. One hundred whole anthers were used as a check in each test.

The results of these tests are shown in experiment 4 of table 4. No virus was obtained from tissue devoid of vascular strands. However, the virus content of the part containing vascular tissue was apparently very low, as was that of the check. Possibly, young anthers contain less virus than more mature ones.

Seeds and Capsules

As stated previously, in capsules of diseased plants the veins are distinctly swollen and the phloem is necrotic, furnishing evidence of the presence of virus. However, the seeds develop and mature normally so far as determined, though their number may be reduced in some cases.

The tobacco seed contains a straight or a slightly curved embryo which is surrounded by endosperm (fig. 10, *I*). The embryo and endosperm do not appear to be connected with each other. Their cells are packed with nitrogenous storage material. No suspensor was observed in the mature embryo. The hilum, a scar indicating the place of attachment of the funiculus to the ovule, forms a projection on the surface of the seed (fig. 10, *I*). The vascular tissue passes without branching through the hilum region and for a short distance through the parenchyma between the outer and inner layers of the seed coat. The position of the vascular strand is indicated by the raphe on the surface of the seed.

Tobacco seeds are much too small to be dissected for determinations of virus content of their various parts, but tests were made of mature seeds, capsule walls, and placenta. One-half gram of material was used in each test, and the procedure followed was the same as that with pollen.

The results of these tests are shown in experiment 5 of table 4. Virus was obtained from all parts of the capsule, including the seed. The results suggest that the virus concentration in tobacco seeds may be much less than that in beet seeds.

DISCUSSION

The results of experiments reported in this paper show conclusively that in some instances the parenchyma tissue selected from the crown and flowering stalk of beet and from the pith of tobacco stems contained virus. However, in every case the virus content was far below that of adjacent regions in which vascular bundles were present. The exact location of the virus in respect to the parenchyma cells is difficult to determine, since there are at least four possible relations that must be considered and evaluated before definite conclusions can be reached. It is possible that the virus may have been present (1) in undetected vascular strands, (2) in surface contaminants from the phloem of adjacent tissues, (3) in phloem exudate in the intercellular spaces, and (4) in the parenchyma cells themselves.

Since no vascular strands were found in any of the parenchymatous regions examined microscopically, it seems probable that vascular tissue was not an important source of virus in these tests. Also, contaminants probably contributed very little to the total amount

of virus recovered from parenchyma, since the washing to which the carefully dissected sections were subjected would be expected to reduce surface contaminants to such an extent that virus in portions not removed would be difficult to detect by the methods employed. It seems probable, therefore, that at least the larger part of the virus recovered from parenchyma of beet and tobacco was derived either from phloem exudate in the intercellular spaces or from the cells themselves.

Although a special effort was made to select plants that showed no leakage of phloem content into the tissues adjacent to the phloem, obviously it is impossible to determine definitely that no such leakage occurred. For the most part, the parenchymatous regions selected for tests were developed in diseased plants several weeks before they were tested for virus content. It is possible that there may have been leakage of phloem content into the intercellular spaces, especially in the earlier stages of development of the tissue, that could not be detected. The resistance of the virus to inactivation by influences likely to be encountered in intercellular spaces is probably sufficiently high to enable it to persist in active condition in such an environment for several weeks.

Since the virus content of the intercellular spaces cannot be determined by direct methods, the evidence on the question of the relation of virus to parenchyma must of necessity be indirect. Failure of virus to traverse medullary rays in tobacco (*2*) indicates inability to move through at least some types of parenchyma. However, the apparent recovery of virus from the perisperm of the beet seed, a tissue in which there are no intercellular spaces, and the recovery of virus from parenchyma of resistant beets and from parenchyma of tobacco point to the probable occurrence of small amounts of virus in certain types of parenchyma.

Perhaps, on the basis of plant structure, it should be expected that at least a small amount of virus occurs in the parenchyma in regions adjacent to the phloem. The presence of a high concentration of virus in the phloem has been definitely established. If protoplasm of the phloem is continuous with that of surrounding cells through the plasmodesma, probably a certain amount of virus would diffuse through the plasmodesma from the phloem to the adjacent parenchyma cells, the diffusion being, perhaps, accelerated by protoplasmic streaming. The magnitude of such an invasion would depend on the rate of outward movement. The virus content, however, would be determined not only by this rate but also by the ability of the virus to multiply in parenchyma and by the rate of inactivation of the virus by parenchyma. Even if it is assumed that all or a large share of the virus recovered from parenchyma is of intercellular origin, the low concentration indicates that the virus either has difficulty in entering parenchyma cells or is inactivated in a short time after coming in contact with the protoplasm of such cells.

The presence of large quantities of virus in the seed of beet and tobacco affords a basis for further speculation regarding the relation of virus to certain types of tissues. Previously (*2*) it was suggested that a tissue barrier exists between the phloem of the mother plant and the young sporophyte. In the beet seed, virus is probably more concentrated in the vicinity of the vascular elements, but it is by no means evident that it is confined to these elements. In fact, the

evidence seems to indicate that some of the virus escapes into regions outside the phloem and perhaps occurs in the perisperm itself. A mechanism that permits an embryo to develop to its full size in the seed and to germinate later, using up a part or all of the food stored in the surrounding tissues, and to remain free from virus, offers indeed an effective barrier to virus movement. No obvious morphologic structure that would account for this phenomenon was detected. The layer of compact tannin-filled cells separating the phloem from the perisperm might be suspected to function in restricting virus to the vascular region. Evidence, however, points to the occurrence of virus on both sides of this layer and suggests that even if virus were able to pass through the tissues surrounding the embryo, including the perisperm, it might still be unable to enter the embryo. Since the embryo develops from a single fertilized cell and grows by absorbing neighboring cells, it may have no protoplasmic connections with adjacent cells, except possibly in the suspensor. If there is no protoplasmic connection, virus would be required to pass out of the adjacent cells through the surface cells of the embryo in order to infect the embryo. In view of the considerable evidence that cell walls, especially those of the epidermis, tracheae, and root hairs, are not permeable to the virus, there seems good reason to suspect that lack of protoplasmic union between the embryo and adjoining tissues would effectively prevent passage of virus into the embryo, even though the perisperm might be thoroughly saturated with virus.

The mechanism that protects from infection the male and female gametophyte and the embryo in its initial development is of considerable interest. Difficulty of invading meristem and parenchyma probably would afford protection to these structures from the curly top virus but additional factors must be operative with such viruses as that of tobacco mosaic, which is undoubtedly able to invade extensively the common types of parenchyma.

If the results of this study indicate relative virus concentration in different parts of the beet plant, the concentration of virus is greater in the seed than in any other part of the plant. Concentrations of virus in the seed greater than those in other parts of the plant could be reached only by multiplication of the virus in the seed or by accumulation against a virus gradient. It is possible that the conditions for multiplication of the virus are very favorable in the developing seed and that the high virus content is produced by multiplication. However, there is no evidence to support this view.

Apparently the increase in virus content of the seed parallels, more or less, the increase of food reserves of the seed. It may be that virus movement is associated in some way with food translocation and that the high virus content of the seed is the result of movement of virus into it from other parts of the plant. In this connection it may be pointed out that mass movement of food materials in the phloem, as suggested by Crafts (*4*) might result in a high virus content in the seed even though no multiplication occurred there. Since virus is present in the phloem it seems reasonable to assume that in a mass movement of liquid content of the phloem into the seed the virus would be carried in also. Of the materials transported to the seed by mass flow, the water would be transpired or utilized, the mineral elements would be utilized or stored, and the sugars would be stored

as starch. Under these conditions, virus would accumulate as a residue and probably would reach a high concentration.

SUMMARY AND CONCLUSIONS

Anatomical evidence indicates that the curly top virus invades the phloem of the entire vascular systems of beet and tobacco. In susceptible varieties of beet the disease is characterized by necrosis of the phloem and hypertrophy and hyperplasia of the phloem and pericycle. Liquid content of the phloem moves through the intercellular spaces of the extraphloem tissue and accumulates on the surface of petioles and leaves.

In plants of the resistant beet variety U. S. 33, anatomical abnormalities in the phloem are less extensive and, so far as observed, very little exudation from the phloem occurs. No anatomical abnormalities were observed in the parenchyma of the ventral side of the petioles or in the pith of the crown and flowering stalk in this variety.

Phloem degeneration in diseased tobacco plants passes through stages similar to those observed in beet. However, the necrotic areas become cavities and are not filled by proliferation of cells adjacent to the necrotic regions, as in beet. No phloem exudate was observed in the intercellular spaces of tissues outside of the phloem, and phloem exudate did not occur on the surface of leaves and petioles. Transverse sections of stems of plants used in these studies appeared normal except for areas of degeneration in the external and internal phloem.

Tests of different tissues of resistant beets indicate a very low virus content in parenchymatous regions of the crown and stalk and in the ventral side of petioles, as compared with adjacent regions containing vascular bundles. Also, much less virus was obtained from wood and pith of tobacco than from bark and from tissue containing internal phloem.

Immature beet seeds have a very low virus content. This content increases as the seed matures, and a very high virus concentration is reached in the mature seed. Apparently this virus is most abundant in the vascular region of the seed, but it is probably not restricted to the phloem. No virus was recovered from the embryo. Seeds heavily charged with virus germinated readily and the developing embryos utilized the food stored in the perisperm without becoming infected. No case of curly top developed in 4,245 plants grown from seed balls produced on sugar beets affected with curly top, tests being conducted before inactivation of the virus in the seed occurred. The virus was inactivated in dry seeds in 3 months.

Virus was recovered from the seeds, capsule wall, and placenta and from all of the flower parts of tobacco. No virus was recovered from pollen or from parts of the anther containing no vascular bundles.

The evidence obtained in this investigation supports the concept that the curly top virus is intimately associated with the phloem of infected plants. The phloem is probably the tissue in which the virus multiplies and it is evidently the tissue in which the virus attains its highest concentration. The exact relation of the virus to tissues other than phloem is uncertain, since it was not possible to determine whether the small amount of virus recovered from the extraphloem regions was derived from the cells themselves or from other sources. However, if virus occurs in parenchyma cells its concentration in such

cells is evidently very low and the conditions are probably very unfavorable to multiplication and spread.

LITERATURE CITED

(1) ARTSCHWAGER, E., and STARRETT, R. C.
1933. THE TIME FACTOR IN FERTILIZATION AND EMBRYO DEVELOPMENT IN THE SUGAR BEET. Jour. Agr. Research 47: 823–843, illus.

(2) BENNETT, C. W.
1934. PLANT-TISSUE RELATIONS OF THE SUGAR-BEET CURLY-TOP VIRUS. Jour. Agr. Research 48: 665–701, illus.

(3) ———
1935. STUDIES ON PROPERTIES OF THE CURLY-TOP VIRUS. Jour. Agr. Research 50: 211–241, illus.

(4) CRAFTS, A. S.
1931. MOVEMENT OF ORGANIC MATERIALS IN PLANTS. Plant Physiol. 6: 1–41, illus.

(5) ESAU, K.
1933. PATHOLOGIC CHANGES IN THE ANATOMY OF LEAVES OF THE SUGAR BEET, BETA VULGARIS L., AFFECTED BY CURLY TOP. Phytopathology 23: 679–712, illus.

(6) ———
1935. ONTOGENY OF THE PHLOEM IN SUGAR BEETS AFFECTED BY THE CURLY-TOP DISEASE. Amer. Jour. Bot. 22: 149–163, illus.

(7) ———
1935. INITIAL LOCALIZATION AND SUBSEQUENT SPREAD OF CURLY-TOP SYMPTOMS IN THE SUGAR BEET. Hilgardia 9: 397–436, illus.

(8) SEVERIN, H. H. P.
1921. MINIMUM INCUBATION PERIODS OF CAUSATIVE AGENT OF CURLY LEAF IN BEET LEAFHOPPER AND SUGAR BEET. Phytopathology 11: [424]–429, illus.

(9) VRIES, H. DE
1879. BEITRÄGE ZUR SPECIELLEN PHYSIOLOGIE LANDWIRTSCHAFTLICHER. CULTURPFLANZEN. VI. KEIMUNGSGESCHICHTE DER ZUCKERRÜBE. Landw. Jahrb. 8: [13]–35, illus.

A MOSAIC DISEASE OF IRIS[1]

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INTRODUCTION

A mosaic disease of bulbous iris was prevalent in California, Oregon, and Washington in the earlier years of commercial plantings in these States. Surveys made during 1928 and 1929 failed to reveal in these States a single large planting that was entirely free from the disease. The percentage of mosaic in original importations from the Netherlands varied widely (from 2 to 90 percent), but there was strong field evidence of spread of the disease after the arrival of the irises in this country, notably in southern California. Some varieties in the older established plantings approached 100 percent infection when the mosaic first came to the attention of the writers. The early presence of the disease in the Netherlands is attested to by Atanasoff.[2]

Preliminary efforts to transmit the disease in 1928–29 were not successful and are not here detailed. The first extensive trials were made in 1929. The senior author left the work in the fall of 1929, the junior author taking it over in the winter of 1929–30 and continuing to the present time. The purpose of this paper is to present the general aspects of the problem, especially the results of studies on the symptoms and transmission of the disease. A preliminary notice[3] of the disease has already appeared.

SYMPTOMS

SYMPTOMS IN BULBOUS IRISES

The symptoms of mosaic in bulbous irises include general dwarfing of the plant, mottling of the leaves, and "breaking" of the flowers. The commercial importance of the disease lies chiefly in the dwarfing effect, which results in a shorter flower stalk for cut blooms and in a lower rate of increase in planting stock. The quality of the flower is also inferior in diseased plants, and the cut blooms do not last as well as normal flowers. Under forcing conditions irises affected with mosaic are conspicuously inferior; in some of the less tolerant varieties they are worthless.

When affected plants are in bud or flower, mosaic mottling of light-green and bluish shades is readily seen and is especially prominent on the leaf bases and bud sheaths. The bud sheath is typically marked with bluish-green blotches on a pale-green ground, or less commonly with yellowish streaks (fig. 1). On immature plants, especially during the early winter growth, mosaic appears as a yellow

[1] Received for publication Apr. 4, 1936; issued December, 1936. Cooperative project of the Oregon Agricultural Experiment Station and the Bureau of Plant Industry, U. S. Department of Agriculture; experimental work conducted at Corvallis, Oreg.

[2] ATANASOFF, D. MOSAIC DISEASE OF FLOWER BULB PLANTS. Izv. B'lgarsk. Bot. Druzh. (Bull. Soc. Bot. Bulgarie) 2: 51–60, illus. 1928.

[3] BRIERLEY, P., and MCWHORTER, F. P. A MOSAIC DISEASE OF BULBOUS IRIS. (Abstract) Phytopathology 24: 4. 1934.

FIGURE 1.—Mosaic mottling in a bud of Imperator iris.

streaking of the leaves. Yellow streaking due to cold injury or to bulb rots also appears at this stage, and diagnosis of mosaic is very uncertain until mottling shows in the new growth. Leaf symptoms are similar in varieties of bulbous irises of all classes, including all flower colors, but the flower symptoms vary. In plants affected with mosaic the younger leaves and bud sheaths are always more plainly mottled than the older. The typical mottling of young leaves (fig. 2, *C*) serves to distinguish the symptoms of mosaic from a distinctive whitish square pattern (fig. 2, *D*) appearing in older leaves of varieties subject to winter injury. This winter-injury pattern is not transmissible. Typical mosaic mottling can be produced on winter-injured plants by inoculating the virus into a variety subject to the latter type of injury.

Flower breaks are usually darker than the normal color of the flower. For example, broken flowers of blue Imperator develop nearly black marks on a blue ground; those of white D. Haring show a purple mottling on normal white falls; and those of lavender Therese Schwartze exhibit prominent purple blotches. The most characteristic flower marking consists of a series of dark blotches of teardrop design (fig. 2, *A*), the tail of the drop extending toward the throat of the flower. Feather-like cleared streaks, lighter than the normal flower color, are associated with mosaic in yellow varieties. The occasional ribbon-like striping of flower parts does not seem to be associated with a virus. This pattern occurs in both healthy and diseased plants and is not transmitted with the mosaic when inoculations are made from plants showing both mosaic symptoms and the striping pattern.

Variations in flower reaction are often observed in a group of individuals of a given variety inoculated with mosaic. Flowers on some plants may be typically blotched on both standards and falls, while those on other plants show few or no markings. An example of this variation in flower response to mosaic inoculation is shown in table 1. The following record may be cited as a further example: Sixteen plants

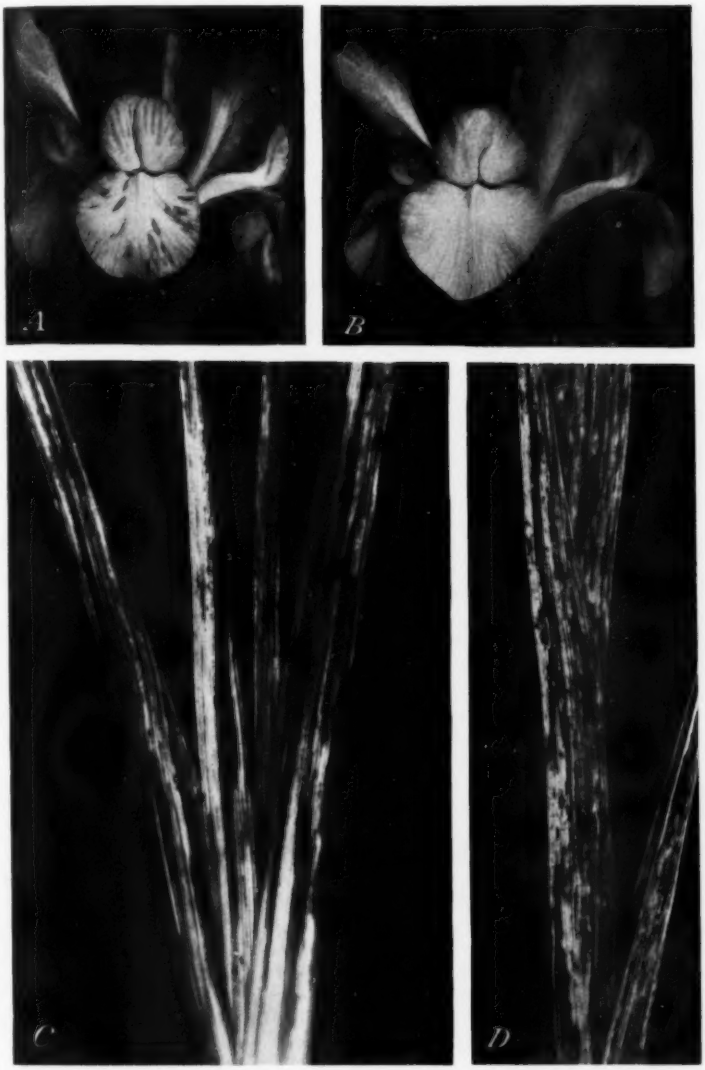

FIGURE 2.—Symptoms of mosaic in bulbous iris: *A*, Diseased flower of Therese Schwartze showing characteristic "teardrop" markings; *B*, normal flower of Therese Schwartze; *C*, yellow-green stripe mottling characteristic of mosaic in young leaves of David Bles; *D*, white and green angular mottling caused by winter injury in Rembrandt (contrasted with *C*).

of the Therese Schwartze variety that exhibited mottled leaves and mottled flowers in 1931 were dug, divided into planting units of one to four bulbs each, replanted, and left in place for 3 years. This subdivision involved 39 clumps. Of these, 19 bore only mottled flowers every year; 12 bore normal (unmottled) flowers in some years; and 8 bore some normal and some mottled flowers on the same clump in some years. There was no apparent correlation between yearly climatic variations and flower symptoms. Successful subinoculations have been made from plants showing typical leaf mottling but no flower symptoms, as well as from plants exhibiting both leaf and flower patterns. This suggests that more than one virus may be involved in the disease herein called iris mosaic, but the evidence is not yet adequate to permit a definite conclusion on this point. In general the symptoms of inoculated plants agree with those of the source plant, severe mosaic resulting from severe and mild mosaic from mild.

TABLE 1.—*Effect of different symptom complexes on height attained by the Dutch iris Therese Schwartze in 1934* [1]

Type symptoms	Type symbol	Cases developed	Mean height of plants and probable error
		Number	*Cm*
Leaves streaked; flowers strongly mottled, with elliptical breaks	1S	29	38.8±0.88
Leaves streaked; flowers average mottled, with elliptical breaks	1A	22	51.9±1.40
Leaves streaked; flowers mildly mottled, with elliptical breaks	1T	5	56.2
Leaves streaked; flowers strongly darkened, with ribbonlike stripes	2	1	70.0
Leaves streaked; flowers apparently normal	3	3	59.0
Healthy iris, inoculated but not infected	H	46	68.6± .57

[1] Uniform stock inoculated by hypodermic needle with standard Therese Schwartze mosaic inoculum in 1933.

The symptom most consistently associated with mosaic is the tendency to a reduction in size and length of shoots. Table 1 furnishes examples of the dwarfing effect associated with mosaic symptoms of different degrees of severity in a uniform lot of Dutch irises. Uniform round bulbs of healthy Therese Schwartze were selected and planted in the fall of 1932. In the spring of 1933 the resulting plants were inoculated by the hypodermic-needle method with uniform composite juice inoculum from a number of mosaic-affected Therese Schwartze plants. The inoculated plants were left undug in the field, and the reaction and measurements of height shown in table 1 were recorded in the progeny in the spring of 1934. The entire block of 106 plants was inoculated, the healthy plants listed in the table representing the individuals that escaped infection.

Analysis shows that plants of the strongly mottled type 1S and of the average mottled type 1A are significantly shorter than those of the healthy class, and that type 1S is shorter than type 1A. No conclusions can be drawn in regard to the other symptom complexes mentioned in table 1, because of the small number of these types (1T, 2, and 3) that appear in the experiment.

SYMPTOMS IN NONBULBOUS IRISES

The symptoms of mosaic in nonbulbous irises are essentially the same as those described for bulbous irises. The thinness of the blade-like leaves of garden forms renders the pale areas more evident, especially by transmitted light.

Many of the dwarf forms of bearded irises show a general mottling that does not exhibit cleared areas. Transmission tests with these are still in progress, but the evidence thus far obtained suggests that not all such mottling is of virus origin. The symptoms in William Mohr, reputed to be a cross between white-bearded Parisiana and *Iris gatesii* Foster, a giant-flowered member of the Oncocyclus section, are of particular interest because of the popularity and high price of this variety. All representatives of William Mohr that the writers have seen are stunted and during the period of active growth are strongly mottled. Yellow, somewhat diffuse, chlorotic areas are developed in some individuals but not in all. Inoculations from this variety to bulbous irises produced a mild mottling but one definitely related to the virus disease here discussed.

CELLULAR CHANGES

There are few morphological features that seem to be related to the activities of the mosaic virus within the tissues of the types of irises studied. The cell arrangement in a section of iris leaf affected with mosaic agrees perfectly with that in a similar section from a healthy leaf. Two gross tissue changes usually accompany external symptom expression: (1) Reduction in number of chlorenchymatous cells and mature chloroplasts in areas beneath the epidermis where extreme mottling is visible, and (2) reduction in size of the epidermal cells of leaves that have been materially shortened by mosaic infection. The reduction in number of chlorenchyma cells and chloroplasts is not always apparent and is unreliable as a criterion of virus infection. The reduction in size of epidermal cells is surprisingly constant and is easily demonstrable by photographing at the same magnification epidermal peelings from healthy and from diseased plants, as shown in figure 3.

Dufrenoy [4] used mosaic-affected iris material in reaching his conclusions and generalizations relative to the changes induced in cells in response to virus infection. A study of several varieties of bulbous irises and of a few nonbulbous forms indicates that the cellular details vary greatly with the variety, the age of the leaf, and possibly with seasonal changes. The vacuolation processes and differentiation of visible byproducts, including X-bodies, do not keep pace with the production of prominent external symptoms. For example, sections of obviously diseased Imperator leaves collected in December 1934 and of young and old leaves of mosaic-affected Therese Schwartze collected in December 1934 and January 1935 showed agglutination of foreign material and strong vacuolation in only one of many leaves examined. In May 1935, during the flowering season, a number of varieties were studied by fixing leaf epidermal strips in acetoformalin, staining in light green, and passing them through dioxan into Canada balsam. In such mounts the epidermal cells are not broken, and their

[4] DUFRENOY, J., and DUFRENOY, M. L. CYTOLOGY OF PLANT TISSUES AFFECTED BY VIROSES. Phytopathology 24: 599–619, illus. 1934.

contents remain in place. The writers have repeatedly checked this technique with living material and find that it leaves visible cell entities produced through virus action in a condition directly com-

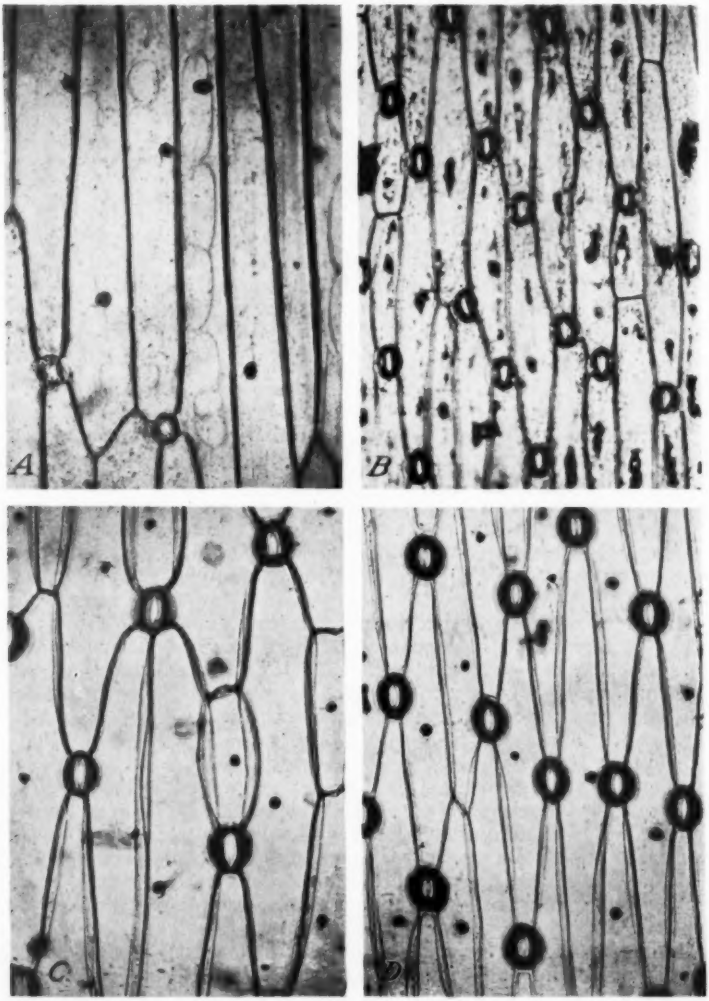

FIGURE 3.—Reduction in size of epidermal cells in iris affected with mosaic: *A*, Healthy Hart Nibbrig; *B*, diseased Hart Nibbrig. Note smaller cells and increased number of visible byproducts in diseased tissue. *C*, Healthy *Iris ricardi*; *D*, diseased *I. ricardi*. Note smaller cells in diseased tissue. × 150.

parable with but far more readily discernible than those in living material mounted in isotonic solutions. With this method it was found that at blooming time the epidermal cells of mosaic-diseased

individuals of some varieties are filled with visible byproducts, while comparable epidermal cells of other varieties remain indistinguishable from healthy ones. Hart Nibbrig in particular is a good variety for study, since it regularly assumes the virus mode of cell disfiguration (fig. 4).

Iris ricardi Hort. during late spring bore strongly mottled leaves when diseased, but the epidermal cells contained normally hyaline protoplasts. Six months later the cell content of aged foliage exhibited more visible byproducts and, in a few instances, partly organized X-bodies. Even in Hart Nibbrig there is little uniformity in the content of morbid cells of comparable leaves from different individuals; there is also some variation, not attributable to age, in similarly located cells of different leaves of the same plant.

The ultimate cellular inclusions, known as X-bodies, are usually not well formed in irises. The vacuolate type is sometimes formed

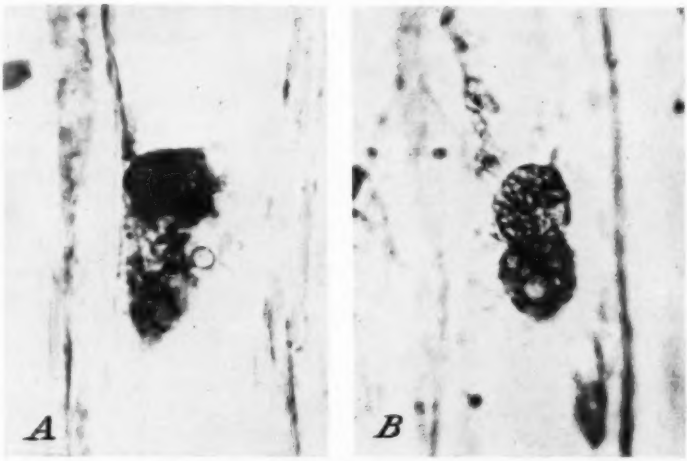

FIGURE 4.—Two types of X-bodies observed in iris affected with mosaic. *A*, Reticulate type in *Iris ricardi*; *B*, Vacuolate type in Hart Nibbrig. Nucleus above in each case. × 1,400.

in epidermal cells of Hart Nibbrig. With a 1.40 N. A. lens and critical illumination these vacuolate types may be resolved into short line-like components. These lines seem to represent the edges of almost ultramicroscopic crystals concentrically arranged around the vacuoles (fig. 4, *B*). The X-bodies within iris cells usually consist of agglutinations of elongate particles which Dufrenoy considers of mitochondrial origin. They are found in cells where the vacuolate type of X-bodies is uniformly absent (fig. 4, *A*).

The epidermal cells of flecked areas in mosaic-diseased flowers contain fewer plastids than corresponding areas of healthy flowers. Comparable areas of diseased and healthy flowers of Therese Schwartze were found to contain 59 and 169 plastids, respectively. This difference is far more noticeable than the similar difference that may obtain in chlorenchyma cells.

Examination of the epidermal cells of the rectangular mottled areas resulting from winter injury in the Rembrandt variety (fig. 2, *D*) reveals more visible particles than the average iris epidermis. The nuclei of these cells are very large, elongate, frequently dumbbell-shaped, and surrounded on all sides by agglutinations of particles. The virus-diseased cell picture differs from this in that the nuclei appear normal and the agglutinated material is adnate to one end or side of the nucleus or present in some other part of the cell.

In the opinion of the writers, internal cell differentiation would be an unreliable criterion for the detection of mosaic in irises. When such differentiation occurs it may be prominent, as Dufrenoy maintains, but it is of quite different form from that induced by fungi that attack iris leaves.

TRANSMISSION TESTS

MECHANICAL METHODS

The mechanical methods used in the experimental transmission of mosaic and the results obtained during five growing seasons are shown in table 2.

TABLE 2.—*Mechanical transmission of iris mosaic*

Season	Method	Total trials	Positive trials	Plants inoculated	Plants infected	
		Number	*Number*	*Number*	*Number*	*Percent*
1929-30	Wedge insertion	38	29	310	103	33
1930-31	do	12	8	74	48	65
	Hypodermic needle	3	1	5	4	80
	Wedge insertion	1	1	11	10	91
	Hypodermic into nodes	34	19	449	60	13
	Hypodermic into stems	1	0	13	0	0
	Hypodermic into leaves	2	0	23	0	0
1931-32	Hypodermic into bulbs	1	0	17	0	0
	Cutting flower stalks	2	0	36	0	0
	Cutting ovaries	2	0	26	0	0
	Handling with contaminated hands	1	0	18	0	0
	Samuel's method	1	0	12	0	0
	Wedge insertion	5	5	45	39	87
	Hypodermic into nodes	2	0	28	0	0
	Hypodermic into internodes	2	2	28	14	50
	Hypodermic into sheathing leaves	2	2	26	13	50
	Hypodermic into internode, node above not cut	1	1	8	4	50
1932-33	Hypodermic into internode, node above cut	1	1	9	5	56
	Hypodermic into internode, internode above cut	1	1	9	7	78
	Hypodermic vertically into pith of cut stem	2	1	17	3	18
	Multiple needle through mosaic-diseased leaf	1	0	8	0	0
	Multiple needle through mosaic-diseased juice	1	0	12	0	0
1933-34	Hypodermic into internodes	4	4	53	38	72

According to the method of inoculation by wedge insertion, a wedge-shaped sliver is cut from the stem of a plant affected with mosaic and inserted into a simple longitudinal slit in the stem of a healthy plant. The wound is then covered with a wrapping of raffia or with a commercial wound dressing known as Tree Seal, to prevent excessive drying. This method was adapted from work with tulip mosaic. It proved useful in cross inoculations between varieties and in determining the significance of different classes of mosaiclike symptoms.

For studying properties of the virus, a method of injecting freshly extracted diseased juice into the stems of healthy plants by means of a fine hypodermic needle (Luer syringe no. 27) was developed by the junior writer. This procedure yielded erratic results in earlier trials but performed consistently after the site of most effective inoculation was determined.

Other methods of inoculation have consistently failed. These included (1) cutting the flower stalk of a healthy iris with a knife used for cutting a diseased flower stalk, (2) cutting healthy ovaries with a knife used for cutting ovaries of diseased plants, and (3) rubbing leaves of healthy irises with the hands contaminated from bruising leaves of diseased plants. These trials indicate that iris mosaic is not likely to be spread from plant to plant in the field in the course of usual operations such as weeding, disbudding, or cutting flowers. No transmission resulted from multiple needle inoculations similar to Sein's,[5] whether the needles were forced through mosaic-diseased juice flooded on the healthy leaf or through a diseased leaf superimposed on the healthy leaf. Samuel's [6] method of rubbing diseased juice into a healthy leaf by means of a roughened glass rod was also without effect.

Inoculations were made during the spring, while the irises were in bud or flower, and records were made from the progeny the following spring. For example, in the growing season of 1929–30 inoculations were made in May 1929 and results were recorded in May 1930. Rows of sister-bulb controls derived from the same clump of the previous year as were the inoculated, bulb for bulb, were alternated with the inoculated rows in the same bed. Controls on all the inoculations listed remained healthy the following season. Either the three largest bulbs from the progeny of each inoculated and each control bulb were saved and grown for observation the following year, or the bulb clumps were left undug and observed in place.

In the seasons of 1929–30 and 1930–31 a number of inoculation trials listed as negative were attempts to transmit mosaic from plants showing atypical symptoms which the results indicated were not mosaic. The percentage of plants infected in these seasons was calculated from the positive trials only. During the seasons of 1931-32 and 1932–33 the inoculum was derived from known mosaic types, and the negative results therefore point to unfavorable factors in technique. The percentage of infection for the trials of these years is based on the total number of plants inoculated.

Before the inoculations of the season of 1931–32 were begun, hypodermic injections of blue "script" ink were made into iris stems in the hope that the regions more readily penetrated by the ink would prove the more effective points for inoculation. It was found that the pith of the internodes readily absorbed a large amount of ink, but that the spread from this region was not rapid. Spread of the blue stain from the point of insertion was most extensive from node injections and from the midribs of leaves injected near the point of insertion on the stem. Since leaf injections were cumbersome, it was decided to use node injection as a standard technique for the

[5] SEIN, F., JR. A NEW MECHANICAL METHOD FOR ARTIFICIALLY TRANSMITTING SUGAR-CANE MOSAIC. Jour. Dept. Agr. Puerto Rico 14: 49–68. 1930.

[6] SAMUEL, G. SOME EXPERIMENTS ON INOCULATING METHODS WITH PLANT VIRUSES, AND ON LOCAL LESIONS. Ann. Appl. Biol. 18: 494–507, illus. 1931.

season. The results recorded in 1932 indicated that the penetration of ink was not a good index of efficiency of inoculation, very low proportions of infection resulting from mosaic-diseased juice injected into nodes. Diseased juice injected into internodes in 1932 yielded much more favorable results, indicating that the internode is the most effective site for inoculation.

TRANSMISSION BY APHIDS

Since aphids were commonly found associated with apparent spread of mosaic in bulbous irises, transmission trials with these insects were undertaken in 1929. The original collections (except no. 76) were first colonized on healthy tulips under cloth cages in the greenhouse. The names [7] and sources of the aphid stocks used are given in table 3.

TABLE 3.—*Aphid stocks used in transmission tests*

Stock no.	Species	Source	
		Plant	Locality
11	*Illinoia solanifolii* (Ashm.)	Potato	California.
71	do	Iris	Grants Pass, Oreg.
77	do	Tulip	Corvallis, Oreg.
54	*Myzus circumflexus* (Buckt.)	Lily	Portland, Oreg.
10	*M. pelargonii* (Kalt.)	[1]	Corvallis, Oreg.
75	do	Lily	Do.
50	*M. persicae* (Sulz.)	Tulip	Do.
53	do	Calla	Do.
51	*Rhopaloniphoninus tulipaella* (Theob.)	Tulip	Salem, Oreg.
76	*Anuraphis tulipae* (Boyer)	Iris [1]	Corvallis, Oreg.

[1] Greenhouse.

The cages used were of 40-mesh muslin on wood frames designed to cover two rows (20 bulbs) planted in Dutch beds. The cages were 2 feet high and had an opening at each end of the top surface which could be closed with a string tie when not in use. These cages were set in place in April before aphids were present on the irises. Sister-bulb controls, exactly comparable bulb for bulb to the caged sets, were grown uncaged, alternated with the sets caged for transmission trials. All progenies from these control plants remained healthy in 1930. Progenies from 29 healthy plants caged with mosaic-diseased plants in the absence of aphids during the 1929 season remained healthy in 1930. The uniform freedom from mosaic of these controls and of the lots of irises exposed to nonviruliferous aphids (table 5) shows that the cases of positive transfer of the virus (table 4) are attributable to the aphids named and that these aphids acquired the virus from the plants designated.

[7] Determined by P. W. Mason, of the Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture.

TABLE 4.—*Transmission tests with aphids caged on mosaic-diseased irises and allowed to migrate to healthy irises under the same cage*

Aphid species and stock no.	Healthy iris variety exposed	Mosaic-diseased iris source of inoculum	Plants exposed in 1929 surviving in 1930	Mosaic-diseased plants, 1930	
				Number	Percent
Illinoia solanifolii, no. 11	Imperator	Imperator 104–1	13	2	15
Do	Hart Nibbrig	Hart Nibbrig	6	5	83
Do	Imperator	David Bles 163	31	3	10
Do	King of Yellows	King of Yellows	10	7	70
Do	King of Whites	King of Whites	3	3	100
Do	Therese Schwartze	Therese Schwartze	16	11	69
Do	Imperator	*Iris ricardi*	12	1	8
Do	do	*I. unguicularis alba*	2	1	50
Illinoia solanifolii, no. 71	do	Imperator 104–1	14	0	0
Do	King of Whites	King of Whites	4	1	25
Illinoia solanifolii, no. 77	Imperator	David Bles 163	11	5	45
Myzus persicae, no. 50	do	Imperator 104–1	16	11	69
Do	King of Whites	King of Whites	5	5	100
Myzus persicae, no. 53	Imperator	Imperator 104–1	11	1	9
Do	do	David Bles 163	18	8	44
Myzus pelargonii, no. 10	do	Imperator 104–1	14	0	0
Do	do	David Bles 163	12	0	0
Myzus pelargonii, no. 75	do	Imperator 104–1	17	0	0
M. circumflexus, no. 54	do	do	11	0	0
Do	do	David Bles 163	11	0	0
Rhopalosiphoninus tulipaella, no. 51	do	Imperator 104–1	15	0	0
Do	do	David Bles 163	13	0	0

TABLE 5.—*Transmission tests in which aphids were transferred by camel's-hair brush from healthy and mosaic-diseased plants to healthy irises*

Aphid species and stock no.	Healthy iris variety exposed	Source of aphids	Plants exposed in 1929 surviving in 1930 [1]
Illinoia solanifolii, no. 11	Imperator	Tulip, healthy [2]	13
Do	do	Tulip, mosaic diseased	16
Do	do	Imperator 104, mosaic diseased	56
Do	do	David Bles 163, mosaic diseased	43
Do	Adrian Backer	Imperator 104, mosaic diseased	20
Do	D. Haring	do	15
Do	Frans Hals	do	12
Do	Golden Glory	do	15
Do	Leonardo da Vinci	do	18
Do	Queen Wilhelmina	do	18
Do	do	David Bles 163, mosaic diseased	17
Do	Rembrandt	Imperator 104, mosaic diseased	17
Do	S. Rombout	do	19
Do	Therese Schwartze	do	19
Illinoia solanifolii, no. 71	Imperator	Tulip, healthy [2]	17
Do	do	Tulip, mosaic diseased	13
Do	do	King of Whites, mosaic diseased	17
Illinoia solanifolii, no. 77	do	Tulip, healthy [2]	16
Myzus persicae, no. 50	do	do	12
Do	do	Imperator 104, mosaic diseased	16
Myzus persicae, no. 53	do	Tulip, healthy [2]	11
Do	do	Imperator 104, mosaic diseased	12
Myzus pelargonii, no. 10	do	Tulip and iris, healthy [2]	19
Do	do	Imperator 104, mosaic diseased	15
Do	D. Haring	do	18
Myzus pelargonii, no. 75	Imperator	Tulip, healthy [2]	10
Myzus circumflexus, no. 54	do	do	14
Do	do	Imperator 104, mosaic diseased	18
Rhopalosiphoninus tulipaella, no. 51	do	Tulip, healthy [2]	10
Do	do	Imperator 104, mosaic diseased	18
Anuraphis tulipae, no. 76	do	Forced iris, mosaic diseased (?)	14

[1] All exposed plants remained healthy in 1930.
[2] Nonviruliferous control.

Two types of transmission trials were made, with strikingly different results. Where aphids were colonized on diseased irises in the same cage with healthy irises and were allowed to migrate to the healthy plants, positive transfer of the virus resulted in several trials (table 4); but when aphids were transferred by means of a camel's-hair brush from diseased irises to healthy irises, there was no transmission (table 5). Similar failure of aphid vectors to transmit a virus when manually transferred had resulted from extensive tests of *Illinoia solanifolii* and *Myzus persicae* with tulip mosaic in 1928–29. It is a common experience to find manual transfer of aphid vectors less efficient than natural migration. This is usually attributed to some injury to the mouth parts of the insect in dislodging it from the leaf. Hoggan [8] has shown that the brush method is of low efficiency as a means of transferring *M. persicae* as a vector of cucumber mosaic, although the aphids were apparently not injured by the brush.

Transfers were made to healthy tulips under cloth cages in the field to serve as nonviruliferous controls on tulip mosaic studies and as general stocks for both tulip and iris tests. Subcultures from these colonies were placed on healthy irises, as a test of freedom from the iris mosaic virus, and other lots were colonized on irises affected with mosaic. Such colonies on diseased irises were allowed to feed and multiply for at least 2 weeks before further transfer to healthy irises for testing.

It is evident from table 4 that *Illinoia solanifolii* and *Myzus persicae* can act as vectors of iris mosaic. Three stocks of *I. solanifolii* transmitted mosaic to 32 percent, 6 percent, and 45 percent of the plants exposed; two stocks of *M. persicae* transmitted mosaic to 76 percent and 31 percent of the plants exposed. *M. pelargonii*, *M. circumflexus*, and *Rhopalosiphoninus tulipaella* failed to transmit iris mosaic in any test, although the diseased plants used as sources of inoculum were comparable to those used in the positive trials with the two vectors mentioned above, and in some trials these species thrived on the diseased plant and migrated to healthy plants to an extent sufficient to make the tests seem adequate. In one trial *Anuraphis tulipae* was transferred from forced Dutch irises containing some mosaic to healthy irises but did not multiply on the latter; similar manual transfers of the two species capable of acting as vectors (table 5) indicate that this test was entirely inadequate.

The great variation in efficiency of *Illinoia solanifolii* and *Myzus persicae* in the several trials shown in table 4 seems explainable largely by the amount of natural migration from the diseased to the healthy plants. In the long, narrow cages used it was hardly likely that aphids would move naturally to all the plants caged unless the infestation was very heavy. Where natural migration was slow, aphids were moved to more distant plants under the cage in some instances, but the results of manual transfer in other tests (table 5) indicate that such moving was not likely to be effective.

Twenty-eight collections of aphids from the leaves and flower buds of bulbous irises included five species [9] distributed as follows: *Illinoia solanifolii* from 15 iris plantings (Washington, 3; Oregon, 10; California, 2); *Myzus persicae* from 5 (Washington, 1; California, 4);

[8] HOGGAN, I. A. SOME FACTORS INVOLVED IN APHID TRANSMISSION OF THE CUCUMBER-MOSAIC VIRUS TO TOBACCO. Jour. Agr. Research 47: 689–704, illus. 1933.

[9] Determined by P. W. Mason.

Macrosyphum sp. from 4 (Washington, 2; Oregon, 1; California 1); *Anuraphis tulipae* from 3 (California); *Aphis* sp. from 1 (California). All collections from Washington bulb plantings were made in mid-May of 1928. California collections were made in late February and early March 1929. All collections in Oregon were made during May, except that *I. solanifolii* was once taken from iris at Grants Pass, Oreg., on February 1. The two species of aphids that have been shown to be vectors of iris mosaic appear to be sufficiently common and widely distributed on bulbous irises, especially when these are in bud, to account for the natural spread of the disease. A paper by Whitaker [10] confirms and extends some of the writers' findings with respect to aphids as vectors of iris mosaic.

HOST RANGE

The bulbous irises here discussed include varieties derived from the species *Iris filifolia* Boiss., *I. tingitana* Boiss. and Reut., *I. xiphium* L. (Spanish iris), and *I. xiphium praecox* Hort. (Dutch iris), all of which are included in the section Xiphium. Cross inoculations between varieties ascribed to these species have been readily successful. In the trials that have been made the wedge-insertion and hypodermic methods and the two aphid vectors transmitted mosaic from one species to another as freely as from plant to plant within a variety. The varieties named below are Dutch irises unless otherwise designated.

Imperator was successfully inoculated with mosaic from Celestial, David Bles (Tingitana type), Hart Nibbrig, Huchtenburg, Imperator, King of Whites (Spanish), J. W. de Wilde, Leonardo da Vinci, Therese Schwartze, Wedgewood (Tingitana), and Yellow Queen. Mosaic from Imperator was successfully transferred to Adrian Backer, Celestial, Golden Glory, Hart Nibbrig, Huchtenburg, Hobbema, Leonardo da Vinci, S. Rombout, Rembrandt, Queen Wilhelmina (Spanish), and Yellow Queen. Inoculations also were successful from David Bles (Tingitana) to Hobbema and Leonardo da Vinci, and from King of Whites (Spanish) and Therese Schwartze to Golden Glory. The failure of one attempt to transmit mosaic from The First (Tingitana) and of several attempts to transmit from David Bles (Tingitana) was probably due to the difficulty of distinguishing mosaic symptoms from other confusing patterns in varieties derived from this broadleaved species rather than to any difficulty in transmitting the mosaic virus. All commercial varieties of bulbous irises tested have been found susceptible, but differences in degree of injury are apparent.

In the season of 1929–30, mosaic was successfully transmitted to Imperator from *Iris ricardi* (section Pogoniris) and from *I. unguicularis* Poir. var. *alba* Hort. (section Apogon). *Illinoia solanifolii* was colonized on potted plants of these species, and the pots were set under cages with healthy Imperator. The resulting mosaic in Imperator has been subinoculated to bulbous irises in later seasons and appears to be the same disease that was found in the bulbous types. The original collections of mosaic-diseased *I. ricardi* and *I. unguicularis alba* were from Redlands, Calif. Attempts to transmit a virus from mosaiclike mottling in German or bearded iris hybrids have not

[10] WHITAKER, W. C. THE POSSIBILITY OF INTER-TRANSMISSION OF TULIP MOSAIC BY INSECT VECTORS, ESPECIALLY APHIDS. Thesis, Oreg. State Col., 26 pp. 1931. [Unpublished.]

been uniformly successful, but mosaic was transmitted to Therese Schwartze from strongly mottled William Mohr in 1934–35.

All attempts to transmit iris mosaic by inoculation into plants outside the genus and all attempts to infect irises with viruses from other genera have failed. These cross inoculations included iris mosaic to Turkish tobacco by leaf rubbing, to tomato and petunia by the carborundum method, and to tulip by wedge insertion and by *Illinoia solanifolii* (manual transfer); tulip mosaic to iris by *I. solanifolii* (natural migration); and lily mosaic to iris by wedge insertion and by hypodermic injection.

DISCUSSION

As pointed out in the description of symptoms, there is great variability in both type and intensity of the disease in inoculated irises. This variability is expressed in the degree of dwarfing and the intensity of flower break and of leaf mottling. In some instances the symptoms of a parent plant have been consistently reproduced in its vegetative progeny for two or more seasons. In other individual plant progenies the severity of symptoms, particularly in the flowers, has changed from year to year.

On subinoculation from plants showing strongly mottled leaves and strongly broken flowers with typical teardrop markings the same type is reproduced in the inoculated plants in the majority of cases, but individuals with mottled leaves and unbroken flowers also appear. In several inoculations both types have appeared within the same inoculated group. When inoculations have been made from individuals with typical mosaic-affected leaves but apparently normal flowers the plants inoculated have developed typical broken flowers more frequently than the symptom type used as inoculum. The intensity of the leaf symptoms in inoculated plants is commonly similar to that of the source plant, but in some instances a mild mottling has resulted from a strongly mottled source.

There is little to indicate that the methods of inoculation used will prove selective if a complex of viruses is involved, inasmuch as severe mosaic mottling of the leaves, intense flower breaks, and extreme dwarfing have all appeared in progenies inoculated by the hypodermic-needle method, by wedge insertion, and by the two vectors *Myzus persicae* and *Illinoia solanifolii*. More detailed experimental analysis, involving the use of seedlings, is needed before the nature of the several types of symptoms can be explained.

The movement of the mosaic virus within the group of bulbs developed by a single plant during the growing season is rapid. If a plant is inoculated during the bud or flowering stage in May and the bulbs are dug and separated in July, the progeny are as a rule all affected with mosaic or are all healthy in the following season. In individual plant progenies from 29 of the positive inoculations made in 1929 and recorded in 1930, all members of the group were affected with mosaic in 137 plant units, 6 units segregated into 1 healthy and 1 diseased, and 1 unit into 1 healthy and 2 diseased. Three segregating units were from plants inoculated by aphids, and four from plants inoculated by wedge insertion. The three largest bulbs were saved from each plant inoculated in 1930. The resulting units grown in 1931 were uniformly diseased or uniformly healthy. Although these

records are based on the performance of the larger bulbs in a plant unit, it appears that the progenies of plants infected as late as the last week in May are likely to be uniformly diseased when grown the following season.

SUMMARY

A mosaic disease of iris was found widely distributed in Washington, Oregon, and California as early as 1928 on bulbous irises originally imported from the Netherlands.

The symptoms of the disease include mosaic mottling of the leaves and the bud sheaths, "breaking" of the flowers, and general dwarfing.

Epidermal cells of mosaic-affected leaves are smaller than normal leaf cells, and intracellular inclusions of vacuolate or reticulate types sometimes occur. The number of plastids is reduced in epidermal cells in flecked areas of flowers affected with mosaic.

The virus is transmissible by insertion of a wedge of diseased stem tissue or by injection of diseased juice by means of a fine hypodermic needle. Other mechanical methods of transfer have been ineffective so far as tested.

Illinoia solanifolii and *Myzus persicae* are able to act as vectors of iris mosaic. *M. pelargonii*, *M. circumflexus*, and *Rhopalosiphoninus tulipaella* failed to transmit the virus in parallel tests.

Cross inoculations between different colored varieties in the bulbous irises and between varieties of Dutch and Spanish irises are readily successful.

Mosaic has been transmitted to bulbous irises from naturally infected *Iris ricardi*, from *I. unguicularis alba*, and from the bearded iris William Mohr.

Attempts to transmit iris mosaic to tobacco, tomato, petunia, and tulip and attempts to infect iris with mosaic from lily and tulip have been unsuccessful.

Attention is called to several symptom types included in iris mosaic that have not yet been satisfactorily explained.

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